THE CATABOLISM OF 2-AMINOETHYLPHOSPHONATE BY *BACILLUS CEREUS*

A THESIS

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This thesis embodies the results of research carried out in the Department of Biochemistry of the John Curtin School of Medical Research, Australian National University, from November, 1966 to October, 1969, during the tenure of an Australian National University Scholarship, for which I am indebted to the Council of the University.

STATEMENT

Portions of the work described in this thesis were carried out as a joint venture or in consultation with other workers. The work described in Chapter II and the part of Chapter III which has been published (Rosenberg and La Nauze, 1968) was carried out in collaboration with Dr. H. Rosenberg. Dr. E. Spinner measured and interpreted the infrared spectra, and Dr. T.J. Batterham the proton magnetic resonance spectra of 2-phosphono-acetaldehyde described in Chapter IV. Miss Frances Pearcey carried out the work on the diester, diacetal of 2-phosphonoacetaldehyde also described in Chapter IV. Finally, in the characterization of purified "phosphonatase", described in Chapter V, Dr. D.C. Shaw prepared the tryptic digest and some of the peptide maps, and Mr. L.B. James performed the amino acid analyses. The remainder of the work was carried out by me under the supervision of Dr. H. Rosenberg.

Candidate's Signature:

Julia LaNauze

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My special thanks are due to my supervisor, Dr. H. Rosenberg, for his guidance, enthusiasm and sense of humour which prevailed throughout the course of this work. I am grateful to Professor F.W.E. Gibson for the opportunity to study and gain experience in his department.

I would like to thank Prof. A.F. Isbell, Prof. J.M. Swan, Dr. L.F. Englert, Dr. E. Spinner, Dr. T.J. Batterham and Miss Frances Pearcey for the part each played in the identification of 2-phosphonoacetaldehyde; Prof. Isbell and Dr. Englert kindly provided me with a quantity of this compound synthesized by chemical means, without which much of my work would not have been possible.

I am also grateful to Dr. D.C. Shaw for help with the protein chemistry and to Mr. L.B. James for carrying out the amino acid analyses of the enzyme "phosphonatase". I am indebted to Dr. M.D. Orr and Dr. C.R. Parish for allowing me to benefit from their experiences with acrylamide gels, and giving me access to unpublished methods, and also to Dr. L.D. Quin for making available the manuscript of a paper (Quin and Shelburne, 1969) before publication. Miss Judy McKee kindly identified

the organism, used throughout this thesis, as Bacillus cereus.

Finally, I thank our Head Technicians, Mr. A.B. Howkins and Mr. I.K. Reid, and other members of the technical staff for their co-operation during my stay in this department; the members of the Photography Department for their care in preparing the photographic prints; and Mrs. Carole Withers for her patience and skill in coping with the typing of this thesis.

PREFACE

Throughout this thesis, each Figure and Table is presented on a separate page and follows the page of text on which the first reference to it has been made.

The abbreviations for units of measurement and for semi-systematic and trivial names of common compounds are those recommended by the editors of Biochimica et Biophysica Acta ("Suggestions and Instructions to Authors", 1965). In addition, the following abbreviations have been used :

AEP	2-Aminoethylphosphonate
EP	Ethanolaminephosphate
Mg^{++}	Magnesium ions, etc.
NEM	N-Ethylmorpholine
Pi	Orthophosphate
TEA	Triethanolamine
TES	N-Tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid

SUMMARY

Compounds which possess a direct link between a carbon and a phosphorus atom are known as "phosphonates"; generally, these compounds are extremely resistant to chemical hydrolysis. The first phosphonate to be isolated from natural sources, 2-aminoethylphosphonate, was found in protozoa (Horiguchi and Kandatsu, 1959) and in a sea anemone (Kittredge, Roberts and Simonsen, 1962). The function of this compound is still not known, but it has become evident, since this time, that many lower animals contain 2-aminoethylphosphonate and its derivatives, and that a considerable amount of the phosphorus of these species is present in this form.

Higher plants and animals appear to be unable to cleave the carbon-phosphorus bond and, if this phosphorus is to be returned to the utilizable phosphorus pool in nature, some organisms must be able to carry out its mineralization. Recently, workers from a number of laboratories have isolated strains of bacteria able to catabolize a variety of phosphonates, but none of these workers have demonstrated the cleavage of the carbon-phosphorus bond by cell-free systems.

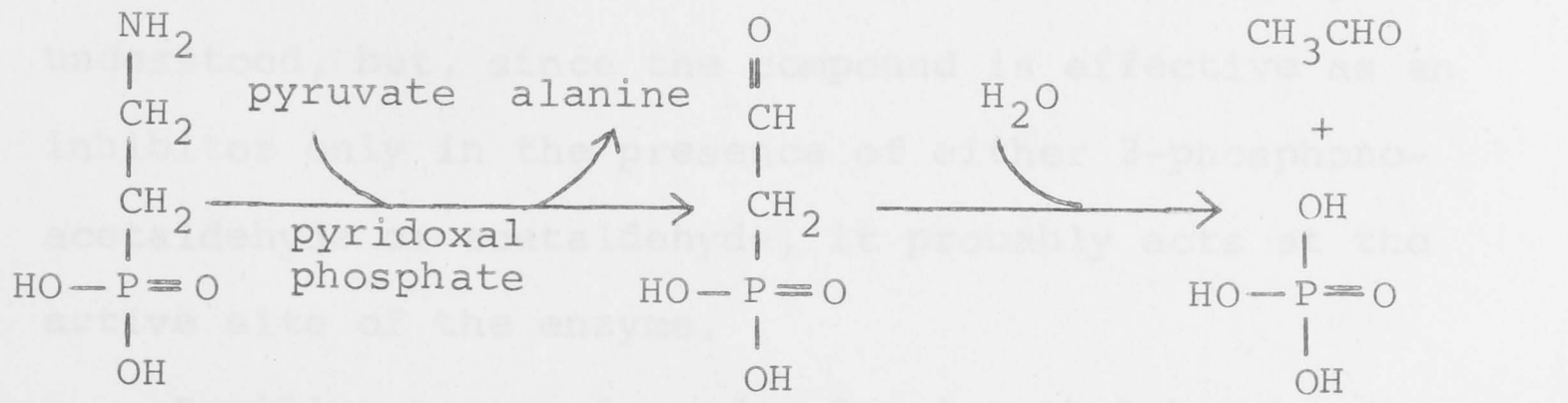
In this laboratory, we have isolated a strain of Bacillus cereus which is able to use 2-aminoethylphosphonate as a sole source of phosphorus. The purpose of the

work described in this thesis was to determine how this organism degrades this compound.

We have found that Bacillus cereus takes up 2-aminoethylphosphonate from the surrounding medium by means of an inducible transport system with a high affinity for this compound. The formation of the transport system, but not its function, is repressed by extracellular orthophosphate; hence the organism shows diauxie when both orthophosphate and 2-aminoethylphosphonate are present in the medium, the orthophosphate being used first. The induction of the transport system is dependent on an energy source and new protein synthesis, since the process is inhibited by 2,4-dinitrophenol, iodoacetate, chloramphenicol and actinomycin D, and requires glucose, oxygen and an amino acid essential for this organism.

Orthophosphite has been found to prevent the growth of whole cells on 2-aminoethylphosphonate, although it is not inhibitory when orthophosphate is the source of phosphorus. Orthophosphite did not affect the uptake of 2-aminoethylphosphonate by the cells, but specifically interfered with its utilization within the cells. Using the specific inhibitory effect of orthophosphite, we have been able to select mutants which are able to grow

on 2-aminoethylphosphonate in the presence of this compound. One of these (AI-2) proved to be very useful in this investigation, since it produced "constitutively" large amounts of the enzymes responsible for the degradation of 2-aminoethylphosphonate to orthophosphate. During the course of breakdown of 2-aminoethylphosphonate by a cell-free preparation from this mutant, a phosphorus-containing compound, other than orthophosphate, was found in the reaction mixture. The addition of orthophosphite to the mixture resulted in an increase in the concentration of this compound. Using this method, we isolated sufficient amounts of the compound to enable us to identify it as 2-phosphonoacetaldehyde on a number of criteria, including comparison with authentic, synthetic material. The following pathway for the degradation of 2-aminoethylphosphonate by B. cereus was subsequently established :



2-aminoethyl-phosphonate

2-phosphono-acetaldehyde

acetaldehyde + orthophosphate

The enzyme (called "phosphonatase"), which catalyses the degradation of 2-phosphonoacetaldehyde to acetaldehyde and orthophosphate, has not been described before. It has been purified and some of its physical and biological properties investigated. It is similar to the alkaline phosphatase of Escherichia coli in a number of ways; for instance, it has a similar pH optimum, it is a dimer of molecular weight of 70-80,000 daltons, and Mg^{++} ions are required for activity. However, it is not an alkaline phosphatase, since it has been found to catalyse the degradation of only one phosphate-ester (p-nitrophenyl-phosphate); conversely, alkaline phosphatase from E. coli was found to be unable to degrade 2-phosphono-acetaldehyde. As mentioned earlier, orthophosphite inhibits the growth of whole cells of B. cereus on 2-aminoethylphosphonate; this compound was found to act by severely inhibiting the activity of phosphonatase. The mechanism of action of orthophosphite is not yet understood, but, since the compound is effective as an inhibitor only in the presence of either 2-phosphono-acetaldehyde or acetaldehyde, it probably acts at the active site of the enzyme.

Bacillus cereus degrades 2-aminoethylphosphonate to orthophosphate by first converting it, via trans-

amination, to 2-phosphonoacetaldehyde; 2-phosphono-acetaldehyde, though still possessing a phosphonate bond, is more labile than 2-aminoethylphosphonate, and is thus more readily cleaved by enzymic attack. It is tempting to suggest that this pathway is adopted universally by organisms which are able to catabolize 2-aminoethylphosphonate; however, since some workers have shown that bacteria can degrade a variety of synthetic phosphonates, other than aminoalkylphosphon-ates, it is evident that alternative pathways, whereby the carbon-phosphorus bond is cleaved, must exist in nature.

TABLE OF CONTENTS

CHAPTER I

General Introduction

The Natural Occurrence and Distribution of Phosphonates

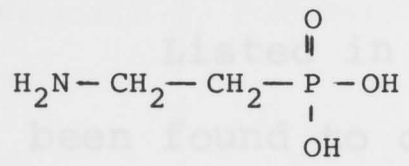
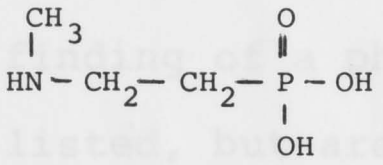
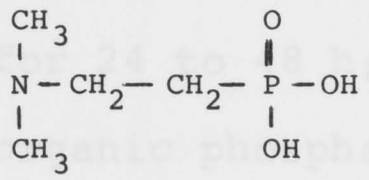
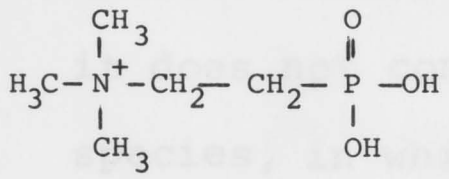
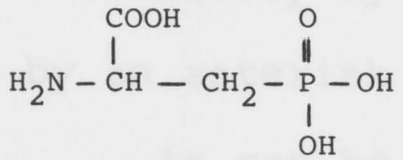
The term "phosphonates" is applied to compounds which possess a covalent link between the carbon and phosphorus atoms. In general, the carbon-phosphorus, or phosphonate, bond is very stable and resistant to both acid and alkaline hydrolysis (Kosolapoff, 1950). However, not all phosphonates share this property, and, as will be discussed later in this thesis (Chapter IV), the presence of functional groups on the alkyl chain of phosphonates can influence their stability (Chavane, 1949; Freedman and Doak, 1957; Maynard and Swan, 1963a, b; Clark, Hutchinson, Kirby and Warren, 1964).

Certain types of phosphonates have been studied extensively for their biological effects, the best known example of which is the group of phosphonates which possess anticholinesterase activity (see O'Brien, 1960). Recently, some phosphonate analogues of nucleotides and other phosphate-esters have been used as enzyme inhibitors (e.g. Myers, Nakamura and Danielzadeh, 1965; Hershey and Thach, 1967; Trentham, 1969). However, although these compounds have pronounced effects on biological systems, they do not occur naturally and are not related to the subject matter of this thesis.

The first demonstration that phosphonates occurred naturally was as recent as 1959, when Horiguchi and Kandatsu (1959) isolated a ninhydrin-positive substance, from acid hydrolysates of rumen protozoa, which they identified as 2-aminoethylphosphonate (AEP) by comparison with material synthesized by chemical means, and named "ciliatine" (Table I.A). These workers found that AEP was not affected by severe hydrolytic conditions, such as treatment with 5 M NaOH at 120° for 8 h (Horiguchi and Kandatsu, 1959) or with 6 M HCl at 120° for 72 h (Kandatsu and Horiguchi, 1962). Methods commonly used for the determination of the total phosphorus content in biological material (Fiske and Subbarow, 1925; Allen, 1940) failed to mineralize AEP; only complete oxidation by heating at 360° in H_2SO_4-$HClO_4$ or H_2SO_4-HNO_3 mixtures was effective (Horiguchi, 1966). Later, these workers reported that 13-15% of the total phosphorus content of the protozoan Tetrahymena pyriformis was present as AEP, and in the same year, Kittredge, Roberts and Simonsen (1962) isolated AEP from the sea anemone Anthopleura elegantissima.

Since this time, compounds containing the phosphonate bond have been found in a wide range of organisms, and several reviews on the topic have appeared (Horiguchi, 1966; Quin, 1967; Kittredge and Roberts,

Table I.A. The structure of naturally occurring phosphonic acids

Structure	Name	Reference to first isolation
	2-Aminoethylphosphonic acid (AEP), also known as "ciliatine"	Horiguchi and Kandatsu (1959) Kittredge, Roberts and Simonsen (1962)
	N-methyl-AEP	Shelburne and Quin (1967) Kittredge, Isbell and Hughes (1967)
	N,N-dimethyl-AEP	Kittredge et al. (1967)
	N,N,N-trimethyl-AEP	Kittredge et al. (1967)
	2-Amino-3-phosphono-propionic acid (phosphonoalanine)	Kittredge and Hughes (1964)

1969; Mastalerz, 1969). So far, the carbon-phosphorus bond has been found only in the form of AEP and its derivatives (Table I.A). Isbell (1967) has synthesized many aminophosphonic acid analogues of amino acids; his synthetic compounds have helped many other workers to identify the naturally occurring derivatives of AEP.

Listed in Table I.B are the organisms which have been found to contain carbon-bound phosphorus. Some of the data in the table do not represent an authenticated finding of a phosphonate in the particular species listed, but are based on the finding of phosphorus which was not mineralized after treatment at 6 M HCl at 120° for 24 to 48 h; under these conditions, all known organic phosphate-esters are hydrolysed, but AEP remains intact. While this method may be a good general approach, it does not constitute positive identification. Those species, in which phosphonates have been "identified" exclusively by this hydrolytic method, have been marked by an asterisk in Table I.B.

As can be seen from Table I.B, the occurrence of carbon-phosphorus in nature is widespread. The presence of phosphonates in certain Annelids, Echinodermates, Arthropods and Chordates cannot be taken to mean that phosphonates are normal metabolites in these organisms,

Table I.B. The occurrence of carbon-bound phosphorus in nature

An asterisk (*) denotes that the particular organism has been reported to contain non-hydrolysable phosphorus, but that this has not been shown to be carbon-bound (see text).

The system of classification is adapted from that given by Miller and Vance (1965).

Kingdom	Phylum	Common name	Reference
Monera	Schizophyta	Five species of bacteria*	Horiguchi (1966)
Protista	Protozoa	Paramaecium $K_3$2*	Horiguchi (1966)
		Rumen protozoa (not specified)	Horiguchi and Kandatsu (1959) Dawson and Kemp (1967)
		Tetrahymena pyriformis	Kandatsu and Horiguchi (1962) Kittredge and Hughes (1964)
		Entodinium caudatum	Dawson and Kemp (1967)
		Seven species of phytoplankton	Baldwin and Braven (1968) Kittredge, Horiguchi and Williams (1969) Rosenberg (unpublished)
	Algae	Euglena gracilis	Rosenberg (unpublished)
Metazoa	Porifera	One sponge*	Quin and Shelburne (1969)
	Coelenterata	Three species of hydrozoa*	Quin and Shelburne (1969) Kittredge and Roberts (1969)
		One jellyfish*	Quin and Shelburne (1969)
		Ten species of sea anemone	Kittredge, Roberts and Simonsen (1962) Kittredge, Isbell and Hughes (1967) Quin (1964, 1965) Shelburne and Quin (1967) Quin and Shelburne (1969) Stevenson, Gibson and Dixon (see text)
		One coral	Kittredge and Hughes (1964)
		One soft coral*	Quin and Shelburne (1969)
		One sea pansy*	Quin and Shelburne (1969)

Table I.B. (cont'd.)

Kingdom	Phylum	Common name	Reference
Metazoa	Annelida	One segmented worm*	Quin and Shelburne (1969)
	Mollusca	Ten species of bivalves (including oysters)	Hori, Itasaka, Inoue and Yamada (1964) Hori, Arakawa and Sugita (1967) Hayashi, Matsubara and Mishima (1967) Quin (1965)
		One oyster* and one scallop*	Quin and Shelburne (1969)
		Spermatozoa from a fresh-water mussel	Higashi and Hori (1968)
		Two species of abalone	De Koning (1966b) Hori et al. (1967)
		One channelled whelk	Quin (1965) Quin and Shelburne (1969)
		One nudibranch	Quin and Shelburne (1969)
		Fifteen species of snails and slugs (aquatic and terrestrial)	Hori et al. (1967) Liang and Rosenberg (1968a) Hayashi, Matsuura and Matsubara (1969)
		One slug*	Horiguchi (1966)
		Two species of cephalopods	Hori et al. (1967)
		One cephalopod (squid)*	Quin and Shelburne (1969)
	Echinodermata	Two species of starfish*	Quin and Shelburne (1969)
	Arthropoda	One shrimp*	Quin and Shelburne (1969)
		One crab*	Quin and Shelburne (1969)
	Chordata	Ox (brain)	Shimizu, Kakimoto, Nakajima, Kanazawa and Sano (1965)
		Cow (milk)	Kandatsu, Horiguchi and Tamari (1967)
		Goat (liver)	Kandatsu and Horiguchi (1965)
		Man (from aotas with atherosclerotic plaques)	Alam and Bishop (1968)

and, as will be discussed later, they are quite likely to have arisen from the ingestion of food containing AEP or its derivatives. Generally, phosphonates are commonly found amongst lower animals, particularly members of the Phyla Coelenterata and Mollusca. Sea anemones have been found to be particularly rich in phosphonates; AEP forms almost 1% of the dry weight of the anemone Metridium dianthus (Quin, 1964), and about 1.8% of the dry weight (or 50% of the total phosphorus content) of the anemone Tealia felina is carbon-bound (Quin, 1965). Because of the difficulty of obtaining many organisms free from symbiotic algae and other microorganisms, the assumption that a particular organism itself is able to synthesize phosphonates is based only on the quantity of carbon-bound phosphorus found in it, and in a few cases, on the rates of incorporation of ^{32}P-labelled Pi into AEP (Liang and Rosenberg, 1968a; Kittredge and Roberts, 1969; Itasaka, Hori and Sugita, 1969). Only in a few instances has the biosynthesis of AEP been demonstrated unequivocally. AEP has been isolated from two species of protozoa grown in pure culture (Tetrahymena pyriformis, Kandatsu and Horiguchi, 1962; Entodinium caudatum, Dawson and Kemp, 1967), and one species of algae (Euglena gracilis, Rosenberg, unpublished observations).

Recently, AEP has also been isolated from pure cultures of several species of marine phytoplankton (Baldwin and Braven, 1968; Kittredge, Horiguchi and Williams, 1969; Rosenberg, unpublished observations). Considering that plankton serve as a food source for many filter-feeding species of marine invertebrates, it is quite possible that AEP may be incorporated into other animals by way of their diet.

A number of vertebrates have also been examined for the presence of phosphonates. Of those living in an aquatic environment (five fishes and one shark; Hori, Arakawa and Sugita, 1967; Quin and Shelburne, 1969), none have contained AEP. However, Shimizu, Kakimoto, Nakajima, Kanazawa and Sano (1965) have isolated small amounts of AEP from bovine brain, and Kandatsu and Horiguchi (1965) found appreciable amounts (58.4 mg) of AEP in the liver of a goat. In these cases, the presence of AEP in the animals is believed to have arisen from the protozoa in their rumen. Kandatsu, Horiguchi and Tamari (1965) also observed the effects of injecting [^{32}P]AEP into rats. They found that, 24 h later, a large portion of the AEP had been excreted in the urine and faeces of the animals, but that a small amount had been incorporated into lipids in the liver. From these

reports, it is evident that humans are also likely to ingest small amounts of AEP by eating meat, dairy products (Kandatsu, Horiguchi and Tamari, 1967) and edible molluscs; Quin (1965) has estimated that a man might ingest as much as 20-30 mg of AEP after eating four clams or eight mussels. It is not known how the human body copes with AEP; work of Hoskin (1956a, b), Kandatsu and Horiguchi and Tamari (described above), and Roberts, Simonsen, Horiguchi and Kittredge (1968) suggests that higher animals are unable to degrade AEP and that it is either excreted in the urine or faeces, or is incorporated intact into lipids. A recent report by Alam and Bishop (1968) described the presence of lipid-bound choline-phosphonate in human aortas with atherosclerotic plaques.

Larvae of the house fly Musca domestica have also been shown to incorporate AEP and methylated derivatives into lipids (Bridges and Rickets, 1966; Bieber, 1968), but no other studies of the natural distribution of phosphonates in insects have been carried out.

Similarly, little attention has been paid to the plant kingdom as a possible source of phosphonates; although Kandatsu and Horiguchi (1962) reported that they found "little or no [AEP].....in yeasts, fungi and plant tissues", they gave no details of their work.

Phosphonates as Components of Phospholipids

In early work (Horiguchi and Kandatsu, 1959; Kittredge et al., 1962), AEP was extracted from natural sources by ether-ethanol or aqueous ethanol mixtures; this suggested that AEP was present in these organisms in association with lipids. Soon after, Rouser, Kritchevsky, Heller and Lieber (1963) reported that they had isolated a new sphingolipid containing AEP from the sea anemone Anthopleura elegantissima. It constituted 9.3% of the total lipid content of the anemone and had completely replaced sphingomyelin (ceramide-choline). The structure of this lipid was confirmed as ceramide-AEP in a later paper (Simon and Rouser, 1967). Kittredge (1967) found that this anemone also contained N-methyl-AEP and N,N,N-trimethyl-AEP in association with lipids, although he did not characterize the particular lipids concerned.

Other workers have since found AEP-containing lipids in a variety of organisms. The identification of these, in a number of cases, has been helped greatly by the chemical synthesis of a number of phosphonic analogues of phospholipids (termed phosphonolipids) by Baer's and Rosenthal's laboratories (for references, see review by Kittredge and Roberts (1969)).

Hori and co-workers (Hori, Itasaka, Hashimoto and Inoue, 1964; Hori, Itasaka, Inoue and Yamada, 1964; Hori, Itasaka and Inoue, 1966; Hori, Itasaka, Inoue, Gamo and Arakawa, 1966; Hori, Arakawa and Sugita, 1967; Higashi and Hori, 1968) have isolated ceramide-AEP from over twenty species of molluscs. In four of the snails examined, they also found a new sphingolipid - ceramide-ethanolaminephosphate - which is the ester analogue of ceramide-AEP; however, they failed to detect this compound in any of the shellfish or cephalopods examined. Hayashi, Matsubara and Mishima (1967) have isolated ceramide-AEP from the viscera of an oyster, and De Koning (1966b) has demonstrated that ceramide-AEP is present in the abalone Haliotis midae. Several lipids, containing derivatives of AEP, have also been found in molluscs : Hori and Arakawa (1969) have isolated a sphingolipid containing N,N-acylmethyl-AEP and N-acyl-AEP from the mussel Corbicula sandai, and Hayashi, Matsuura and Matsubara (1969) have isolated a sphingolipid containing N-methyl-AEP from the snail Turbo cornutus.

AEP has also been found as a constituent of lipids in protozoa. Dawson and Kemp (1967) produced evidence which suggested that ceramide-AEP, phosphatidyl-AEP and glycerol-ether-AEP were present in a pure culture of the

protozoan Entodinium caudatum and in a mixture of protozoans from the rumen. The ratio of phosphatidyl-AEP to phosphatidyl-ethanolaminephosphate was 1:1.1 in E. caudatum and 1:1.7 in the rumen protozoa. Liang and Rosenberg (1966) found that Tetrahymena pyriformis also contained phosphatidyl-AEP, but it was present in this organism in a lower molar ratio to phosphatidyl-ethanolamine (1:13). These workers were also able to show the incorporation, in cell-free systems, of AEP into cytidinemonophosphate-AEP and the synthesis of an AEP-containing glycerophosphatide from cytidinemonophosphate-AEP and dipalmitin; however, for reasons discussed later, these reactions were considered to be either a salvage mechanism for free AEP, or a result of the lack of specificity for base in the normal phospholipid-synthesizing enzymes present in the organism. Carter and Gaver (1967) isolated ceramide-AEP from T. pyriformis and found that the lipid moiety consisted of C_{17} and C_{19} branched chain sphingosines. Thompson (1967), also working with T. pyriformis, found significant amounts of lipids containing glycerol-ether bound to both ethanolamine and AEP. Thompson's findings have now been confirmed by comparison of the natural product with synthetic monoester- and diester-monoether phosphatidyl-AEP (Chaco and Hanahan, 1969).

Phosphonates as Components of Proteins

In addition to its wide distribution in association with lipids, AEP also occurs in association with proteins. Rosenberg (1964) found that AEP was bound to the lipid-free, proteinaceous residue of Tetrahymena pyriformis, and that, even after incubation of the residue for 6 h with proteolytic enzymes, during which time a large portion of the material dissolved, the bulk of the AEP was still associated with the residue. Quin (1964, 1965) reported that 1.1% of the lipid-free proteinaceous residue of the sea anemone Metridium dianthus was AEP. He found that although pepsin effectively solubilized the residue, 90% of the AEP was still associated with one polypeptide which was large enough to be precipitated by trichloroacetic acid. A dinitrophenyl-derivative of the soluble polypeptide failed to yield, on hydrolysis, any dinitrophenyl-AEP, thus indicating that the amino group of AEP was not free in the polypeptide. It is not yet known how AEP is bound to proteins. Quin (1967) suggested that AEP might be present simply as an appendage to the polypeptide chain, or as a cross link between chains, or actually situated in the chain (however, the latter possibility would raise problems of amino acid coding during protein synthesis). A recent

survey by Quin and Shelburne (1969) has shown that protein-bound AEP is particularly predominant in sea anemones. Indeed, in the species examined, between 25 and 45% of the total phosphorus in the proteinaceous fractions was carbon-bound. Recently, Stevenson, Gibson and Dixon (quoted by Kittredge and Roberts, 1969) have purified two proteases from the sea anemone Metridium senile which contain 3.7 and 7.4 residues of AEP, respectively for a molecular weight of 20,000. Unfortunately, at the time of writing, no further details of this work were available.

The Possible Function of Phosphonates

The occurrence in nature of compounds containing carbon-bound phosphorus is widespread, but their function is not yet known. Since some organisms possess considerable amounts of phosphorus in this form, it seems likely that such compounds are of real significance to them : for instance, in some, ceramide-AEP has totally replaced sphingomyelin and ceramide-ethanolaminephosphate, and, as mentioned earlier, 50% of the total phosphorus content of the sea anemone Tealia felina is carbon-bound (Quin, 1965). Animals of lower Phyla, particular sea anemones, contain more carbon-bound phosphorus than higher animals. In general, these animals live in an

aquatic environment where the ability to synthesize phosphonates may be advantageous. It seems possible that phosphonates, which are usually extremely resistant to hydrolysis and are not cleaved by the action of phosphatases (Kittredge, Roberts and Simonsen, 1962), may confer stability to the molecules to which they are attached. Phospholipids are largely constituents of membranes, and it is possible that phosphonolipids may provide the organisms with a particularly resistant cell membrane. Thompson (1969) has found that the phosphonolipids of Tetrahymena pyriformis are resistant to attack by endogenous lipolytic enzymes (thought to be a mixture of phospholipase A and lysophospholipase). As mentioned above, the work of Rosenberg and Quin showed that the AEP-containing portions, of the proteinaceous residues from two unrelated organisms, were both resistant to the action of proteolytic enzymes. On the basis of its natural distribution, Rosenberg (unpublished, personal communication) pointed out that AEP is produced only in species which do not possess a protective covering (such as cellulose, chitin, squamous epithelium or bacterial-type cell walls) on tissues exposed to the surroundings. This was illustrated in the case of two flagellates - Euglena gracilis and Chlamydomonas reinhardi - in which

he found that the former contained AEP, whereas the latter did not. The cell walls of Euglena are unlike those of Chlamydomonas in that they are largely protein, and not cellulose, in composition (Leedale, 1967). In all cases, the species, reported to contain AEP, but not conforming with this hypothesis, are those in which the occurrence of AEP as a natural metabolite is unproven (see earlier). Although this theory is attractive, a wider range of organisms must be tested before it can be verified.

The isolation of two proteases containing AEP from a sea anemone is of particular interest; this is the first instance of the purification of a protein containing AEP, and demonstrates that AEP can occur in association with non-structural proteins. These proteases are secreted by the anemone into its gastrovascular cavity, where the presence of the phosphonic group may make the enzymes stable to the action of other proteases present in the cavity.

The Biosynthesis of the Carbon-Phosphorus Bond

The mechanism whereby the carbon-phosphorus bond is synthesized in nature is not yet known. Earlier, Rosenberg (1964) reported that, when growing cells of the protozoan Tetrahymena pyriformis were given ^{32}Pi, the AEP bound to lipids was labelled much more rapidly

than either free AEP or that bound to protein. These results suggested that, in this organism, a lipid intermediate is involved in the formation of the carbon-phosphorus bond. To account for these observations, Segal (1965) proposed, purely on theoretical grounds, that AEP was synthesized by the rearrangement of phosphatidylethanolamine to phosphatidyl-AEP, or a similar rearrangement of phosphatidylserine to phosphatidyl-phosphonoalanine and subsequent decarboxylation. More recently, workers from four different laboratories have tested a variety of compounds as possible precursors of AEP in T. pyriformis (Trebst and Geike, 1967; Warren, 1968; Liang and Rosenberg, 1968b; Horiguchi, Kittredge and Roberts, 1968). In summary, they found that the hypothesis of Segal was not supported by experimental evidence, since, although both serine and ethanolamine were incorporated rapidly into lipids, the compounds were poor precursors of the carbon skeleton of AEP. Instead, they found that glycolytic intermediates were the best precursors, and that [1-^{14}C], [2-^{14}C] and [6-^{14}C]-labelled glucoses were more efficient in labelling AEP than [3,4-^{14}C]-labelled glucose. Trebst and Geike (1967), by selectively degrading the AEP from the cultures, were able to show that the 1-carbon of AEP was most likely

derived from the 1-carbon (or 6-carbon) of glucose, and that the 2-carbon of AEP was derived from the 2-carbon of glucose. All four groups of workers came to the conclusion that the most probable precursor was phosphoenolpyruvate, which underwent a rearrangement to 3-phosphonopyruvate, and subsequent transamination to 2-amino-3-phosphonopropionic acid (phosphonoalanine) and decarboxylation to AEP (See Fig. I.2, discussed below). The experimental evidence, on which they based their conclusions, has been represented schematically in Fig. I.1.

Warren (1968) was able to detect a very slight incorporation of ^{32}P from ^{32}P-labelled phosphoenolpyruvate into AEP in "broken cell" homogenates. Both he and Trebst and Geike (1967) suggested that phosphoenolpyruvate underwent an intramolecular rearrangement to form 3-phosphonopyruvate (Fig. I.2). However, as pointed out by Liang and Rosenberg (1968b), this scheme involves free AEP as a precursor of lipid-bound AEP, and it is therefore incompatible with the labelling pattern of AEP observed in this organism. These workers suggested, instead, that phosphoenolpyruvate undergoes an intermolecular rearrangement with an ester of phosphatidic acid, and that the carbon, but not the phosphorus, moiety of phosphoenol-

AEP Glucose Phosphoenolpyruvate

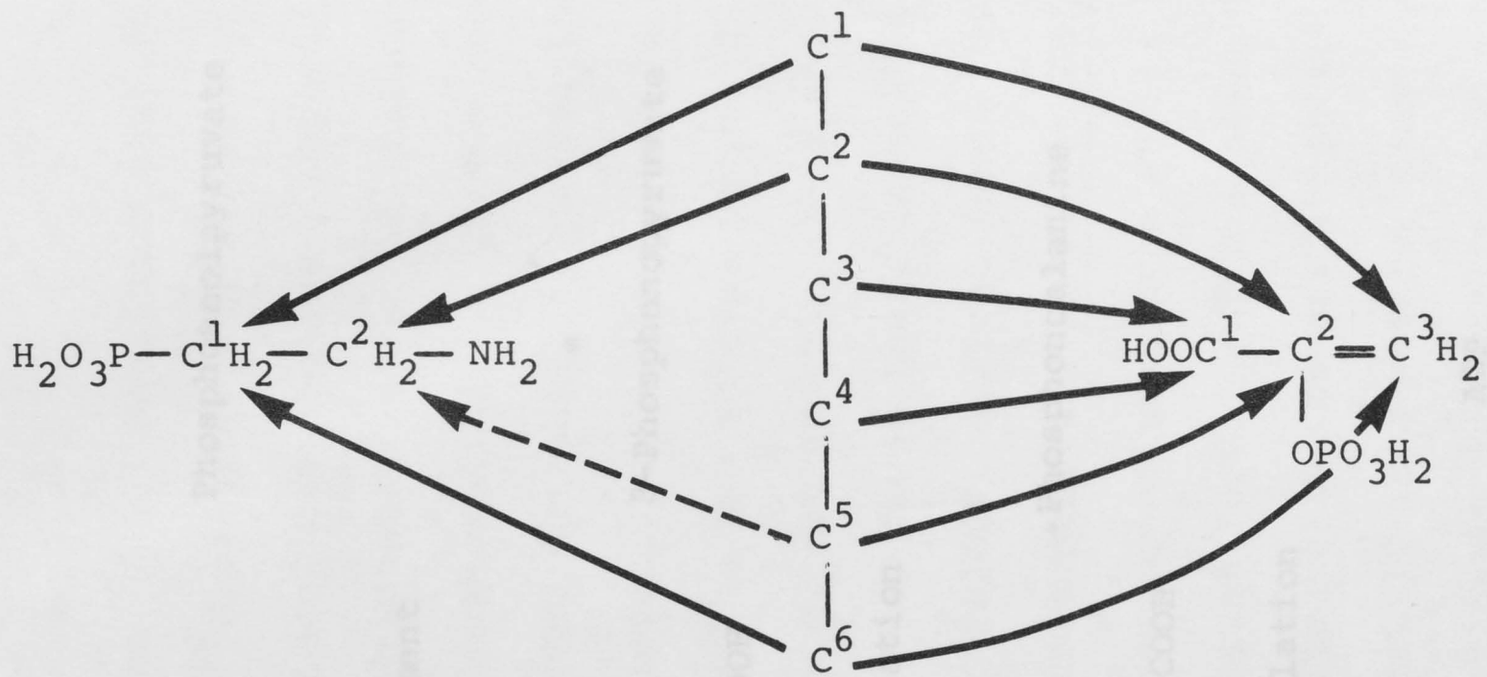


Fig. I.1. Predicted labelling of AEP by respective glucose carbons, assuming that the synthesis of AEP involves phosphoenolpyruvate as an intermediate (see Figs. I.2 and I.3). ⟶ indicates that the pattern of labelling is supported by experimental evidence.

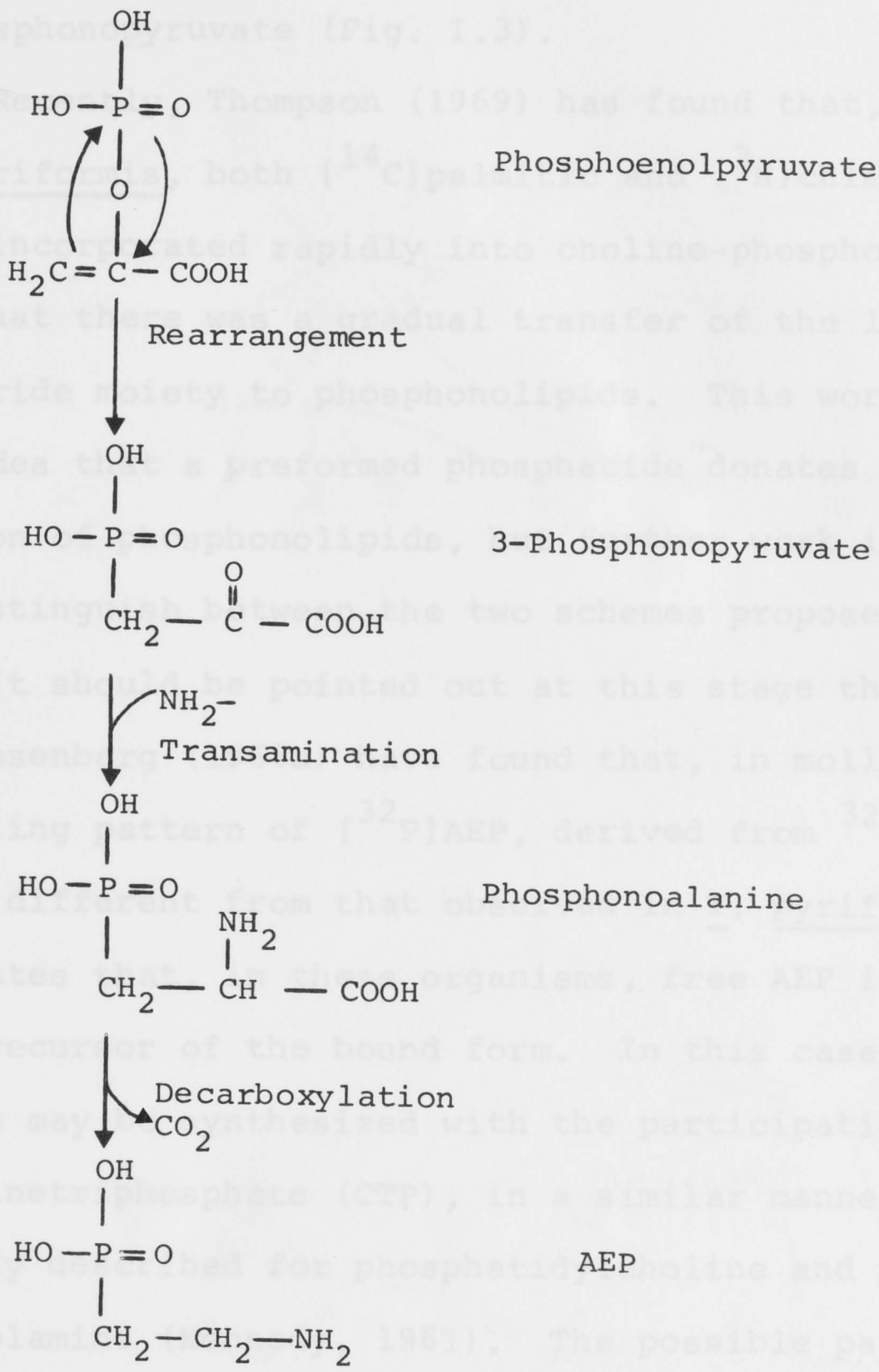


Fig. I.2. Possible route for the biosynthesis of AEP via a rearrangement of phosphoenolpyruvate (according to Trebst and Geike, 1967, and Warren, 1968).

pyruvate is incorporated into a lipid-bound form of 3-phosphonopyruvate (Fig. I.3).

Recently, Thompson (1969) has found that, in T. pyriformis, both [^{14}C]palmitic and [^{3}H]chimyl alcohol were incorporated rapidly into choline-phospholipids, and that there was a gradual transfer of the labelled glyceride moiety to phosphonolipids. This work supports the idea that a preformed phosphatide donates the lipid portion of phosphonolipids, but further work is necessary to distinguish between the two schemes proposed above.

It should be pointed out at this stage that Liang and Rosenberg (1968a) have found that, in molluscs, the labelling pattern of [^{32}P]AEP, derived from ^{32}Pi, is quite different from that observed in T. pyriformis, and indicates that, in these organisms, free AEP is probably the precursor of the bound form. In this case, phosphonolipids may be synthesized with the participation of cytidinetriphosphate (CTP), in a similar manner to that already described for phosphatidylcholine and phosphatidylethanolamine (Kennedy, 1961). The possible pathways which have been proposed for the biosynthesis of phosphatidyl-AEP have been summarized diagrammatically in Fig. I.4.

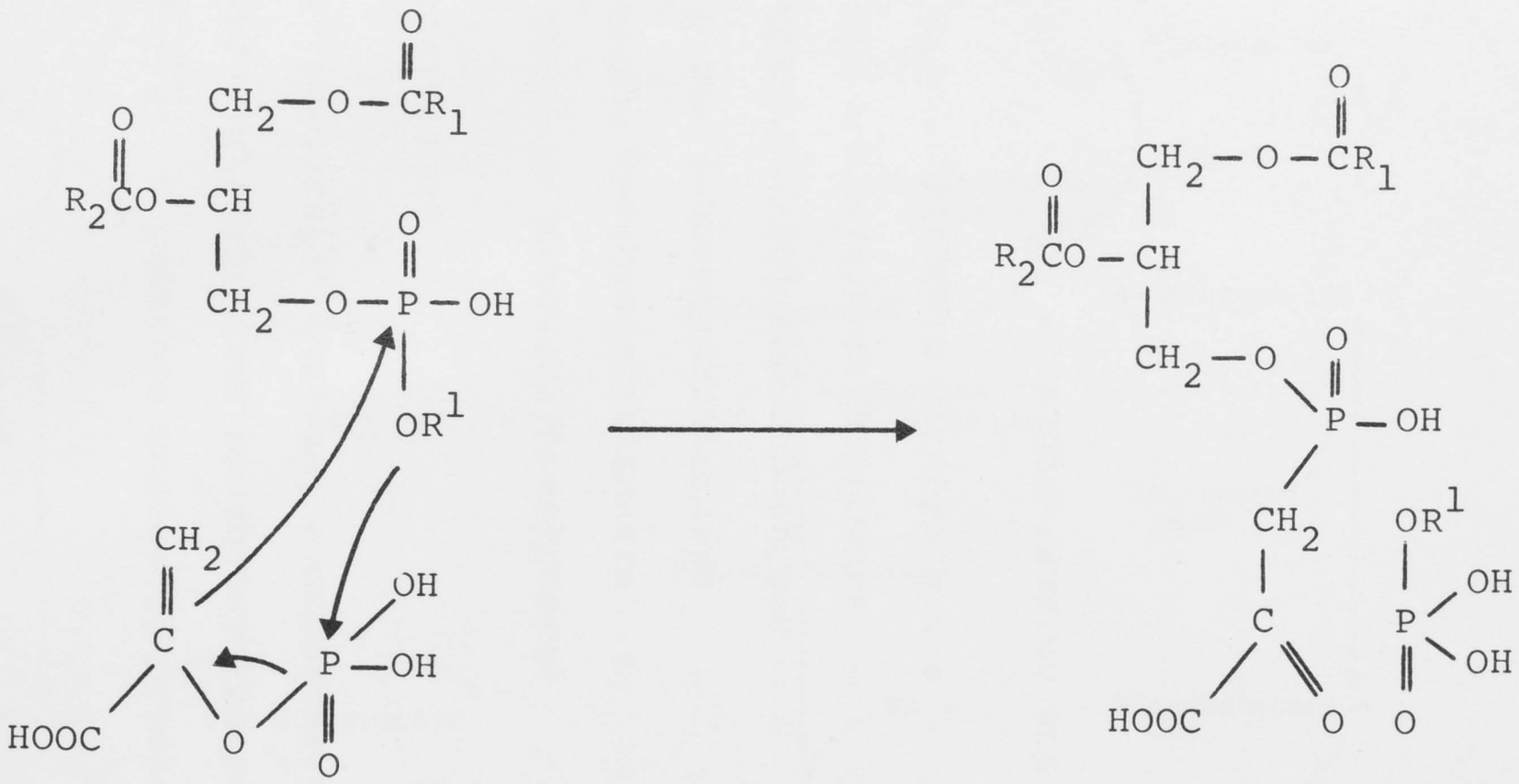


Fig. I.3. Alternative rearrangement of phosphoenolpyruvate to form the glyceride ester of 3-phosphonopyruvate (according to Liang and Rosenberg, 1968b).

Fig. I.4. Possible routes of biosynthesis of phosphatidyl-AEP from phosphoenolpyruvate (according to H. Rosenberg, personal communication).

Abbreviations :

AEP = 2-aminoethylphosphonate

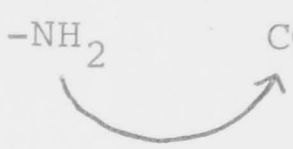
PEP = phosphoenolpyruvate

PPA = 3-phosphonopyruvate

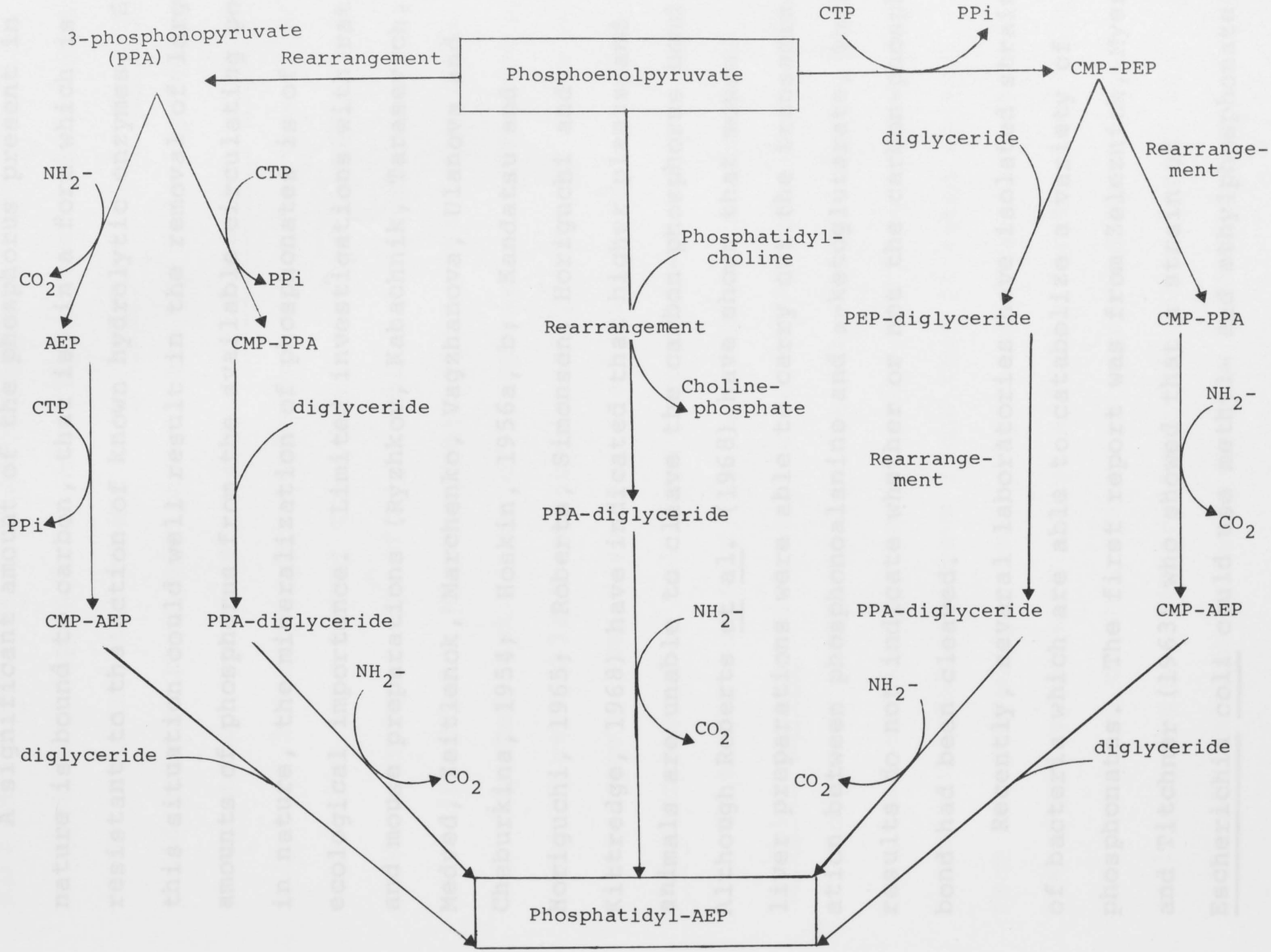
CMP = cytidinemonophosphate

CTP = cytidinetriphosphate

PPi = inorganic pyrophosphate



= transamination and decarboxylation


3-phosphonopyruvate
(PPA)
Rearrangement
Phosphoenolpyruvate
CTP
PPi
CMP-PEP
NH2-
CTP
CO2
PPi
AEP
CMP-PPA
Phosphatidyl-
choline
Rearrangement
Choline-
phosphate
diglyceride
Rearrange-
ment
CTP
diglyceride
NH2-
PPi
PPA-diglyceride
PEP-diglyceride
CMP-PPA
CO2
CMP-AEP
PPA-diglyceride
NH2-
PPA-diglyceride
CMP-AEP
NH2-
NH2-
CO2
diglyceride
CO2
CO2
diglyceride
Phosphatidyl-AEP

The Catabolism of Phosphonates

A significant amount of the phosphorus present in nature is bound to carbon, that is, in a form which is resistant to the action of known hydrolytic enzymes. Since this situation could well result in the removal of large amounts of phosphorus from the available circulating pool in nature, the mineralization of phosphonates is of ecological importance. Limited investigations with rat and mouse preparations (Ryzhkov, Kabachnik, Tarasevich, Medved, Zeitlenok, Marchenko, Vagzhanova, Ulanova and Cheburkina, 1954; Hoskin, 1956a, b; Kandatsu and Horiguchi, 1965; Roberts, Simonsen, Horiguchi and Kittredge, 1968) have indicated that higher plants and animals are unable to cleave the carbon-phosphorus bond. Although Roberts *et al.* (1968) have shown that mouse liver preparations were able to carry out the transamination between phosphonoalanine and α-ketoglutarate, their results do not indicate whether or not the carbon-phosphorus bond had been cleaved.

Recently, several laboratories have isolated strains of bacteria which are able to catabolize a variety of phosphonates. The first report was from Zeleznick, Myers and Titchner (1963) who showed that a strain of *Escherichia coli* could use methyl- and ethylphosphonate

as a source of phosphorus. Since this time, other reports have indicated that many microorganisms may be able to metabolize phosphonates. Mastalerz, Wiezcorek and Kochman (1965) found that a strain of E. coli and of E. freundi could grow on both ethylphosphonate and 2,3-dihydroxypropyl-phosphonate, whereas a strain of Mycobacterium phlei grew only on 2,3-dihydroxypropylphosphonate. Harkness (1966) found that nine out of the ten strains of bacteria tested grew on at least one of eight aminoalkylphosphonates, and recently, Tanabe, Misono, Schichiji and Kandatsu (1969) have reported that 24 out of 62 species of microorganisms examined were able to metabolize AEP. However, none of these reports have described the cleavage of the carbon-phosphorus bond by cell-free preparations.

In this laboratory, we have isolated a strain of Bacillus cereus which is able to use AEP as a sole source of phosphorus. This thesis describes experiments which have been carried out to determine how this organism catabolizes AEP. We have found that it takes up AEP from the surrounding medium by means of an inducible transport system with a high affinity for AEP, and that the formation of this system is repressed by extra-cellular, but not intracellular Pi. Once inside the cell, AEP is transaminated to form 2-phosphonoacetaldehyde

which is degraded, by an enzyme not previously described, to acetaldehyde and Pi. The enzyme carrying out the second reaction has been purified and some of its properties are described.

CHAPTER II

The Transport of 2-Aminoethylphosphonate by *Bacillus cereus*

INTRODUCTION

The chemistry and occurrence of phosphonates in nature has been discussed in detail in Chapter I. Although their function in nature is not yet known, they are by no means rare and probably occur far more commonly than first thought (Quin, 1967; Quin and Shelburne, 1969). The continuous production of phosphonates by a variety of life forms could represent a serious loss of phosphorus from the circulating pool in nature, especially since higher plants and animals appear to be unable to break the carbon-phosphorus bond. Thus, it is of vital importance that other organisms cleave the bond so that the phosphorus is once more available for general use. Recently, a number of bacteria have been found which can use phosphonates as sole sources of phosphorus and carbon (Zeleznick, Myers and Titchner, 1963; Mastalerz, Wieczorek and Kochman, 1965; Harkness, 1966), but nothing was known of the mechanisms they used to degrade these compounds.

Since AEP is the most common phosphonate occurring naturally, it seemed logical to study the metabolism of this compound, rather than that of the synthetic compounds used by other workers. In addition, the ease with which ^{32}P-labelled AEP could be prepared biosynthet-

ically from Tetrahymena pyriformis (Liang and Rosenberg, 1966) was an important consideration. A number of microorganisms available to us were tested for their ability to use AEP as a sole source of phosphorus. One of these was chosen for study as it grew well on AEP, and it was subsequently identified as Bacillus cereus.

The first problem we decided to examine was the mechanism whereby this organism took up AEP from the surrounding medium. Our findings are presented in this chapter. During the course of this work, we found that orthophosphite inhibited the growth of the organism when AEP was the source of phosphorus, and experiments to determine its site of action are also described.

MATERIALS AND METHODS

Chemicals

Amino acids used in media were products of Nutritional Biochemicals Co. and were all of the L-configuration. AEP and other phosphonates were obtained from Professor A.F. Isbell of the Texas A and M University, and were chromatographically pure. Chloramphenicol was a product of Parke Davis; actinomycin D was a gift from Merck, Sharpe and Dohme.

Analytical grade orthophosphite could not be obtained. Various brands of Na_2HPO_3 were examined and all were found to be contaminated with Pi. Finally, recrystallized material was prepared from the least contaminated of these (BDH, Laboratory Reagent). Solutions were prepared freshly before use from the solid material, as stock solutions showed a tendency to oxidize slowly to Pi. Freshly prepared solutions were estimated to contain less than 0.5% Pi.

All other chemicals were either of analytical grade or of the highest purity available.

Labelled Compounds

[^{32}P]Orthophosphate was purchased from the Australian Atomic Energy Commission. [^{32}P]AEP was prepared biosynthetically from Tetrahymena pyriformis

as described by Liang and Rosenberg (1966). The specific radioactivity of the product varied between 0.5 and 1.5 mC/mmole, and for most purposes was diluted with unlabelled AEP before use. [^{3}H]AEP was prepared by an exchange process from 120 mg of crystalline AEP, at the Tritiation Service of the Radiochemical Centre, Amersham. Exchangeable ^{3}H was removed from the crude material by repeated evaporation of aqueous solutions. Chromatographically, radiochemically pure AEP was recovered from this product as described by Liang and Rosenberg (1966). The final yield of pure AEP was 70 mg, with a specific radioactivity of 1.6 μC/mg. This material was dissolved in water to yield a 0.1 M solution which was used without further dilution with carrier. [^{32}P]-Ethanolaminephosphate was prepared as described by Hagerman, Rosenberg, Ennor, Schiff and Inoue (1965). All solutions of radioactive materials were Millipore-filtered before use.

Media

The following media were used :

(1) A complex medium (referred to as PPYG) containing 2% (w/v) proteose peptone (Difco), 0.5% (w/v) yeast extract (Difco) and 1% (w/v) glucose.

(2) A defined, phosphorus-free medium (BXPG-NEM) containing the following amino acids (g/l) : L-alanine, 1.0; L-arginine, 1.0; L-aspartic, 2.5; L-glutamic acid, 2.5; glycine, 0.5; L-histidine, 1.0; L-isoleucine, 1.0; L-leucine, 3.5; L-methionine, 0.5; L-serine, 2.0; L-threonine, 2.0; L-tryptophan, 0.5; L-valine, 2.0; and salts (mg/l) : $MgSO_4.7H_2O$, 1.2; $(CH_3COO)_2Zn.2H_2O$, 0.2; $(CH_3COO)_2Ca$, 43; $CuCl_2$, 3.0. The medium was supplemented with iron in the form of a ferric EDTA chelate to a final concentration of 0.16 M. The pH was adjusted to 7.0 with KOH, and the medium sterilized by autoclaving.

(3) A defined medium (with phosphorus) identical with BXPG, but supplemented with 2.5 mM K_3PO_4 (pH 7) referred to as PG.

(4) A defined medium, identical with BXPG, but with 2.5 mM AEP added as the sole source of phosphorus, and referred to as AG.

Glucose was autoclaved separately as a 20% (w/v) solution and was added to all media to a final concentration of 1% (w/v).

Microorganism

The bacterium used in the present work was one of a number isolated from the gut of the earthworm by Dr.

J.N. Parle, while he was working in this Department. It was chosen because it grew well on AEP as a sole source of phosphorus. It was subsequently identified as Bacillus cereus on 26 criteria (Bergey, 1957) and will henceforth be referred to as B. cereus (W). It was also found to be an absolute valine auxotroph.

Six authentic strains of B. cereus have since been found to grow on AEP. These were B. cereus var. terminalis (T), strains NCTC 7464 and 9946, strain ATCC 14579 and two strains (132 and 133) from the School of Microbiology, University of Melbourne.

Bacillus cereus (W) also had the advantage that it formed spores readily. Stock suspensions of spores are still viable (at the time of writing) after 4 year's storage at 4°. Spores were prepared by growing the organism on AG medium and allowing the suspension to shake for 5 days. By this time, the suspension consisted almost entirely of spores. These were centrifuged, suspended in sterile, distilled water, and heated at 80° for 10 min. The suspension was washed twice with distilled water before it was dispensed in about 2 ml amounts in sterile Bijou bottles.

Uptake Studies

Bacteria were grown overnight at 30° in PPYG medium.

By this time they were in stationary phase and had reached a population density of 1.0-1.3 x 10^9 cells/ml. They were centrifuged and resuspended in BXPG medium diluted with an equal volume of N-ethylmorpholine-HCl buffer, pH 7.5 (referred to as BXPG-NEM). They were then shaken at 250 gyrations/min for 2 h to deprive them of phosphorus; there was no increase in cell number or cell dry weight during this time. At 2 h, they were diluted to a density of 1.0 x 10^8 cells/ml, radioactive substrate added, and the rate of shaking altered to 325 gyrations/min to increase the aeration. At intervals, samples were withdrawn and filtered rapidly on membrane filters (Millipore or Oxoid) which were washed with two 3-ml lots of 0.9% NaCl, dried and pressed into planchettes. The planchettes were counted on a Nuclear Chicago gas-flow counter fitted with a thin Mylar window. Uptake was expressed as mμmoles of AEP per 10^8 cells, using a standard sample counted with each experiment.

In experiments involving mixed nuclides (^{32}P and ^{3}H), the samples were counted on a Packard Tri-carb (series 3000) scintillation counter, and two of the three channels were employed for the simultaneous assay of the two activities. Under the conditions used, ^{3}H produced no counts above background in the channel used

for ^{32}P, while the latter showed a "spillover" of about 20% into the ^{3}H channel. This error was corrected by counting the samples twice, at an interval of 4 weeks and subtracting that fraction of the total radioactivity which was calculated to decay with a half-life of 14.3 days.

Electrophoresis and Radioautography

Electrophoresis on Whatman 3 MM paper was carried out at pH 2.0 in a cooled-plate apparatus (Atfield and Morris, 1961), usually for 25 min with a voltage gradient of 100 V/cm. The buffer used contained 12.4 ml of 98% formic acid and 43.5 ml of glacial acetic acid per 1.

Radioautographs were prepared by exposing the chromatograms for a suitable time interval to Kodak "Blue Brand" film.

RESULTS

Control of AEP Utilization

Bacillus cereus (W) was able to use either Pi or AEP as sole sources of phosphorus when these were added to the defined medium (BXPG). However, when the cells were given both sources of phosphorus at the same time, the pattern of growth was discontinuous, or diauxic (Fig. II.1). The cells used AEP only after all the Pi had been removed from the medium. Further investigations showed that cells which had been grown in PPYG medium and resuspended in BXPG-NEM containing [^{32}P]AEP, began to transport AEP only after more than 2 h had elapsed, suggesting that endogenous Pi was used in preference to AEP. This suggestion was confirmed when cells, which had been deprived of Pi for 2 h (see Materials and Methods), were shown to transport AEP about 50 min after it was presented to them (Fig. II.2). Similarly, cells which had been grown on AG medium and then resuspended in fresh BXPG-NEM medium, took up AEP immediately.

The induction of the transport system depended on new protein synthesis and an energy source, since uptake did not take place when glucose or valine were omitted from the medium, nor when either 2,4-dinitrophenol (0.1 mM), iodoacetate (0.5 mM) or chloramphenicol (10 µg/ml)

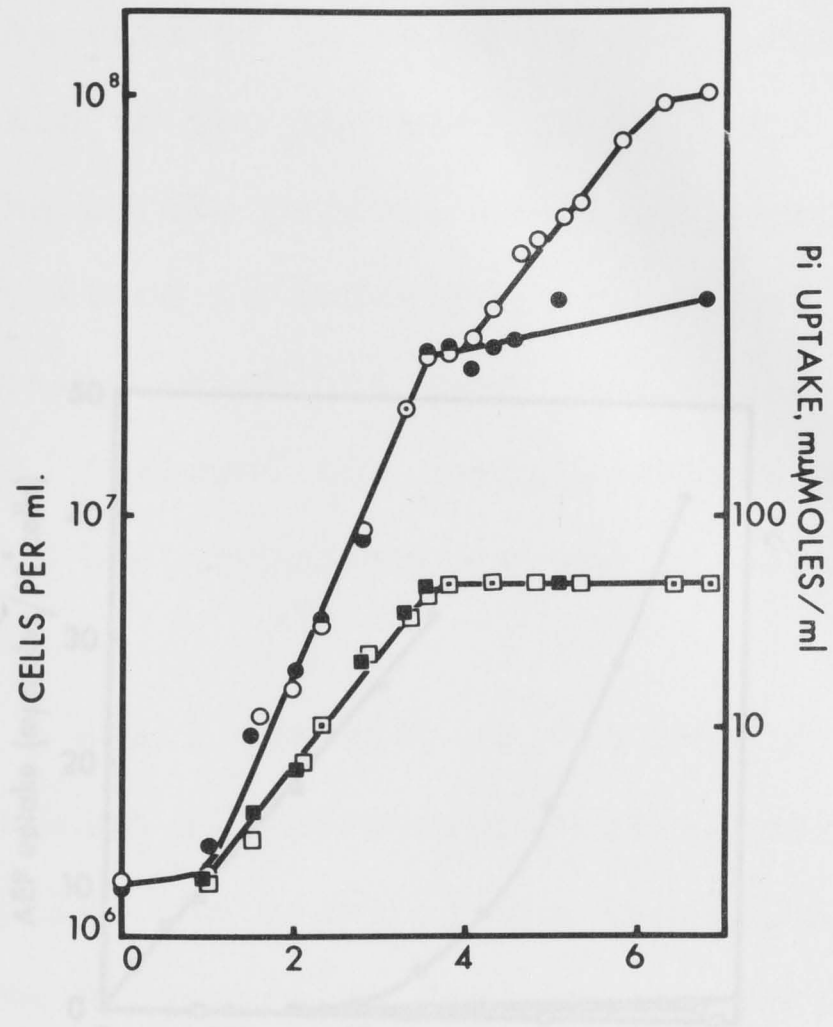


Fig. II.1. The growth of B. cereus (W) on a mixture of phosphorus sources. Stationary phase cells grown on PG medium were inoculated to a density of 1.3 x 10^6 cells per ml into each of two flasks containing BXPG medium and supplemented with the following sources of phosphorus : Flask A contained 0.05 mM ^{32}Pi; Flask B contained 0.05 mM ^{32}Pi and 1 mM AEP. The flasks were sampled half-hourly and the uptake of Pi and cell numbers were determined. Cell numbers per ml : Flask A, ●——●; Flask B, ○——○. ^{32}Pi uptake (mμmole/ml) : Flask A, ■——■; Flask B, □——□ .

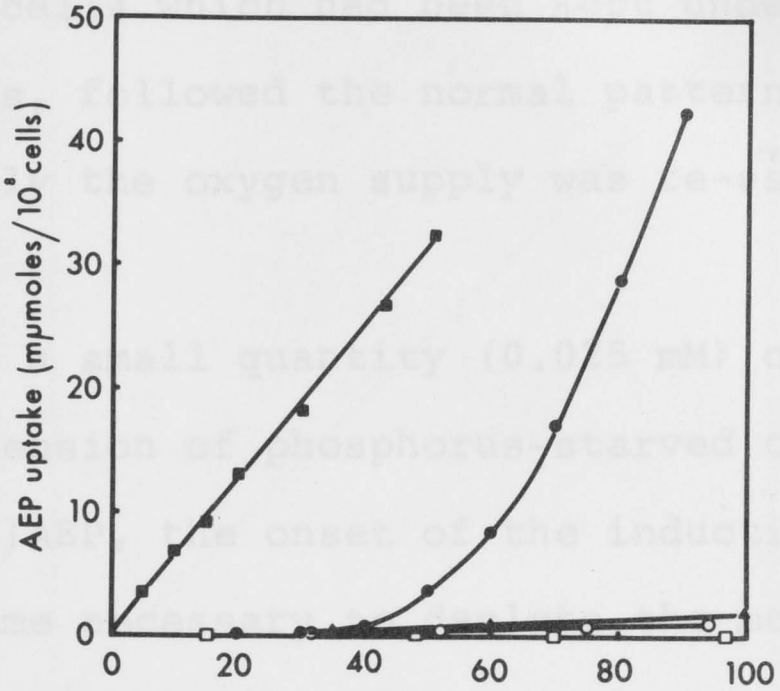


Fig. II.2. Factors affecting the induction of AEP transport in B. cereus (W). Cells were prepared for uptake studies as described in Materials and Methods. They were then shaken in the presence of 0.5 mM [^{32}P]AEP and the rate of uptake followed. The various conditions used were : control, ●——● ; glucose omitted or iodoacetate (10^{-5} M) present or dinitrophenol (10^{-4} M) present, □——□ ; chloramphenicol (10 μg/ml), ○——○ ; valine omitted, ▲——▲ . The line at the left (■——■) represents AEP uptake by cells grown on AEP (AG medium).

was present (Fig. II.2). Following the brief induction period, the rate of increase in AEP uptake bore a linear relationship to the increase in cell dry weight (Fig. II.3). The uptake process was also dependent on a supply of oxygen, since no induction took place in its absence. However, cells which had been kept under anaerobic conditions, followed the normal pattern of induction immediately the oxygen supply was re-established (Fig. II.4).

When a small quantity (0.025 mM) of ^{32}Pi was added to a suspension of phosphorus-starved cells together with [^{32}P]AEP, the onset of the induction was delayed by the time necessary to deplete the medium of the added Pi (Fig. II.5). This result was confirmed by a similar experiment (not shown) where ^{32}Pi and [^{3}H]AEP were used together. In this case, the preferential uptake of Pi before that of AEP was clearly demonstrated, although there was a measure of scatter of the points obtained with ^{3}H because of the necessary correction for the "spillover" of radioactivity of the ^{32}P into the ^{3}H channel (see Materials and Methods).

Although Pi did not interfere with the transport system for AEP once it had been induced (Fig. II.6; cf. Fig. II.14), it did prevent further increase in the

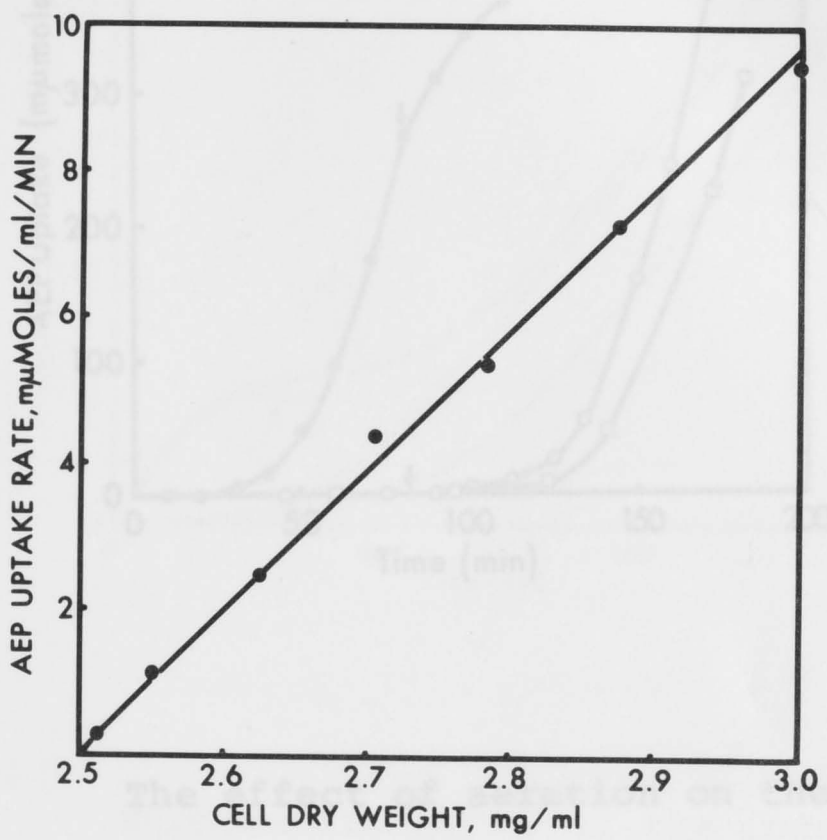


Fig. II.3. The rate of uptake of AEP by B. cereus (W) as a function of growth.

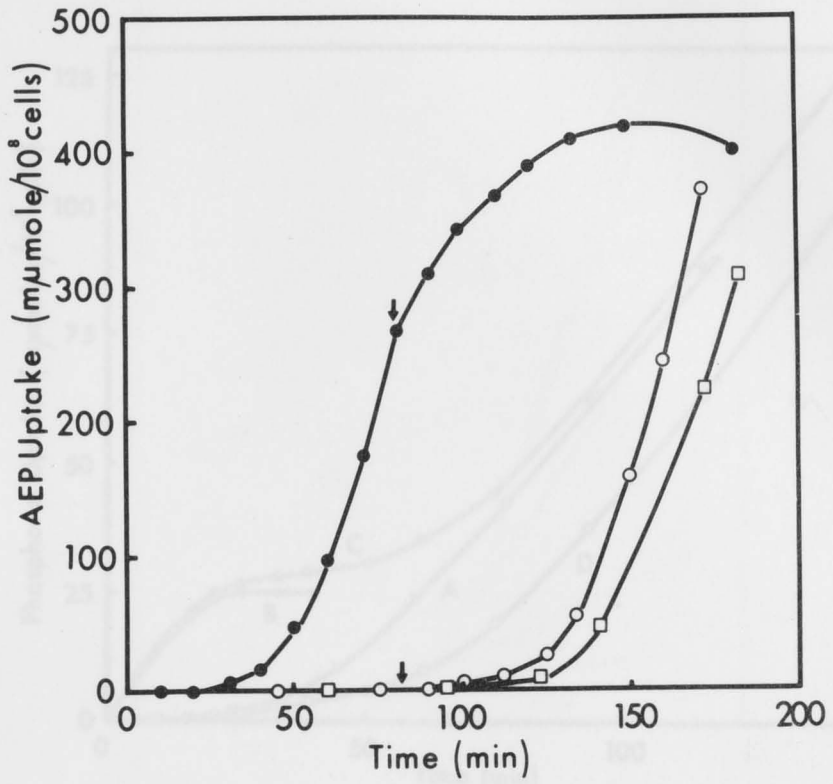


Fig. II.4. The effect of aeration on the induction of AEP transport in B. cereus (W). The cells were prepared for uptake studies as described in Materials and Methods. 0.5 mM [^{32}P]AEP was added to each flask which were treated as follows : Flask A, shaken at 325 gyrations/min, ●——● ; Flask B, not shaken, ○——○ ; Flask C, shaken as Flask A, but under N_2, □——□ . At the time shown by arrows, shaking was stopped in Flask A and aeration (with shaking) begun in Flasks B and C.

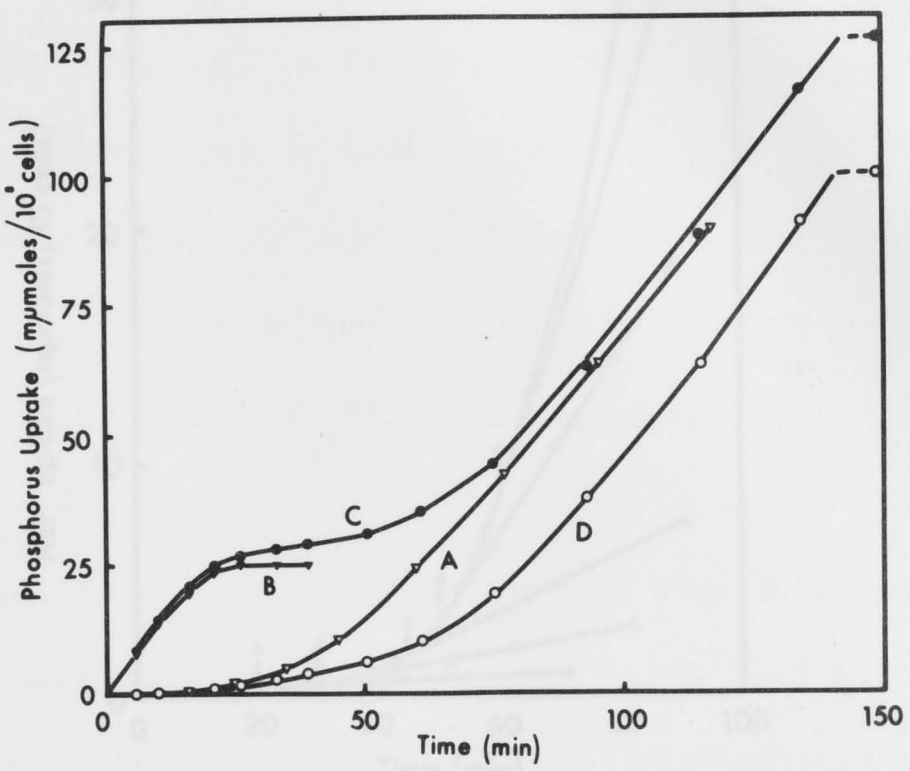


Fig. II.5. Suppression by Pi of the induction of AEP transport in B. cereus (W). The cells were prepared for uptake studies as described in Materials and Methods. They were supplemented with either 0.1 mM [^{32}P]AEP (Curve A, ▽——▽) or 0.025 mM ^{32}Pi (Curve B, ▼——▼) or both (Curve C, ●——●). The uptake of ^{32}P by the cells was followed and the figures expressed as mμmole P, using appropriate standards. Curve D (○——○) was obtained by subtracting Curve B from Curve C.

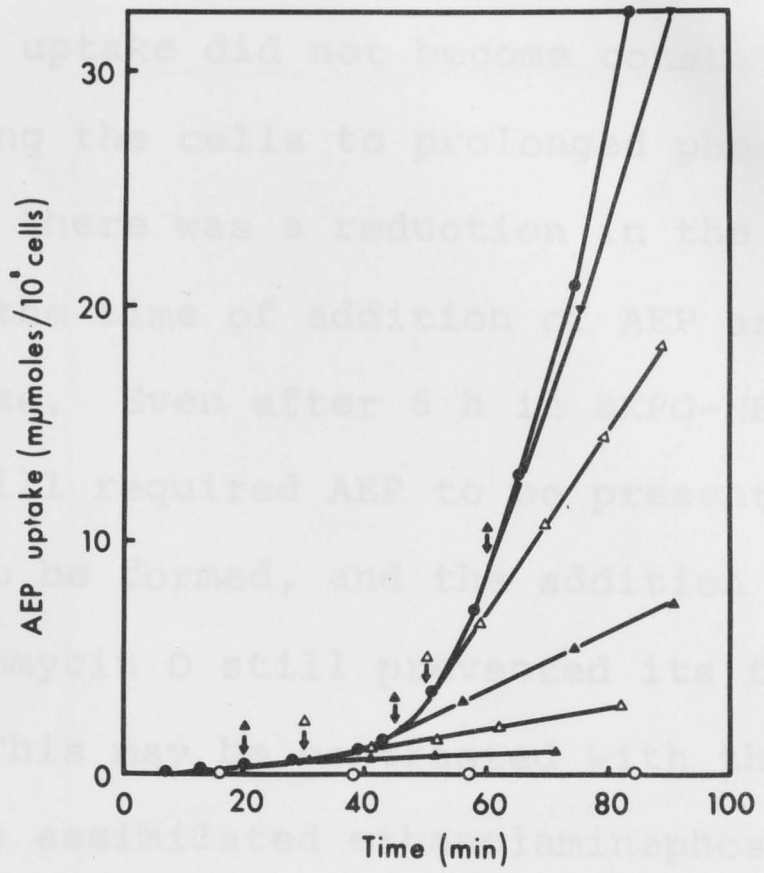


Fig. II.6. The effect of actinomycin D on the induction of AEP transport and the rate of uptake in B. cereus (W). The cells were prepared for uptake studies as described in Materials and Methods. They were shaken in the presence of 0.5 mM [^{32}P]AEP and the rate of uptake followed by sampling (control curve, ●——●). At various times during the incubation, indicated by arrows, portions of the suspension were transferred to pre-warmed flasks containing Pi (▲——▲) or actinomycin D (△——△) at a final concentration of 0.5 mM or 1 μg/ml, respectively. The rate of uptake in all flasks was followed by periodical sampling. The effect of adding either of the two compounds together with AEP at zero time is also shown (○——○).

rate of uptake from the time it was added to the cells. In this respect, it acted similarly to actinomycin D.

AEP uptake did not become constitutive merely by subjecting the cells to prolonged phosphorus-starvation, although there was a reduction in the delay (or lag) between the time of addition of AEP and the beginning of its uptake. Even after 6 h in BXPG-NEM medium, the cells still required AEP to be present for the uptake system to be formed, and the addition of chloramphenicol or actinomycin D still prevented its formation (Fig. II.7). This may be contrasted with the manner in which the cells assimilated ethanolaminephosphate (EP). Cells, grown in PPYG medium and resuspended in BXPG-NEM medium containing 0.5 mM [^{32}P]EP, required an induction period of 40 min before they began to transport the ^{32}P-phosphorus (Fig. II.8). However, cells which had been deprived of phosphorus for 2 h, transported ^{32}P-label earlier and at a faster initial rate. By 4 h, the cells took up ^{32}P-label at a linear rate immediately EP was presented to them. At this stage, the initial rate of uptake was not affected by either chloramphenicol (10 µg/ml) or actinomycin D (1 µg/ml). It is evident that although AEP and EP are structurally related, they are assimilated by entirely different processes in B. cereus (see Discussion).

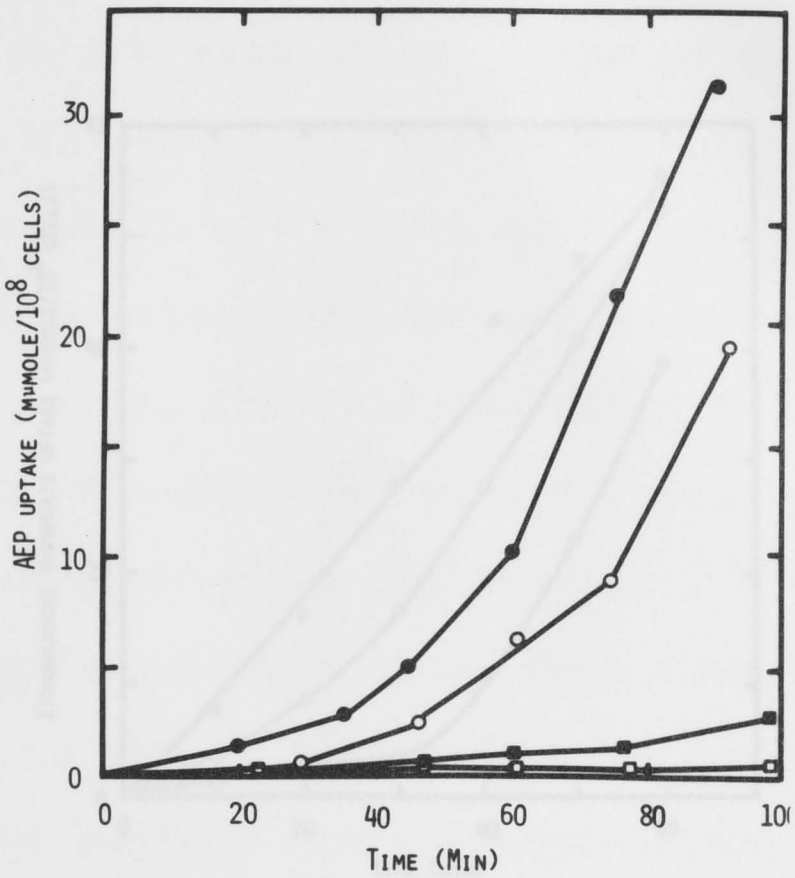


Fig. II.7. The effect of prolonged phosphorus-starvation on the rate of uptake of AEP by B. cereus (W). Cells were grown in PPYG medium as described in Materials and Methods. They were resuspended in the phosphorus-free medium (BXPG-NEM) and deprived of phosphorus for 6 h. At this stage, 0.5 mM [^{32}P]AEP was added and the rate of uptake followed : no other additions (control), ●—● ; chloramphenicol (20 μg/ml), □—□ , or actinomycin D (1 μg/ml) added, ■—■ . The uptake of AEP by the same preparation of cells after only 2 h starvation is shown for comparison, ○—○ .

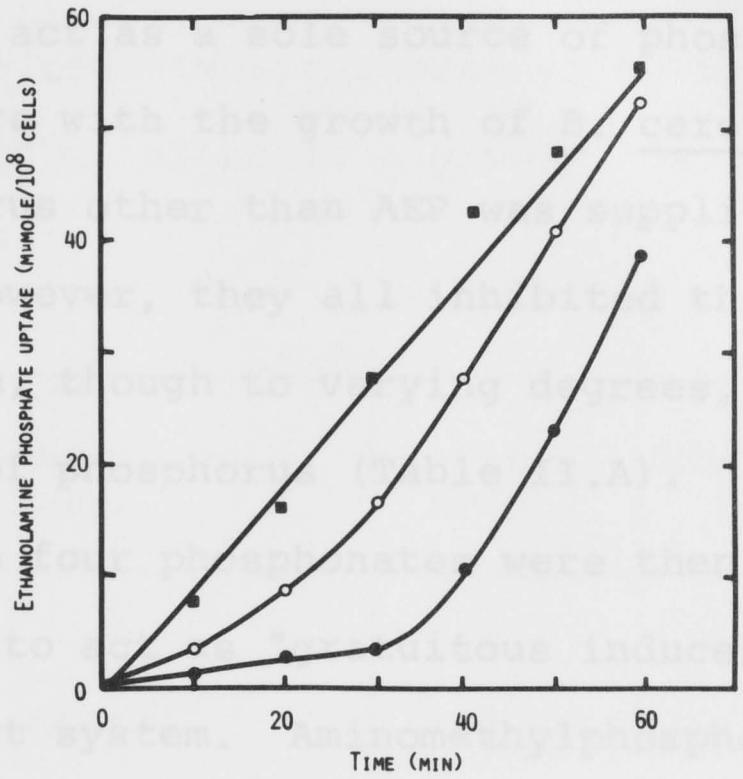


Fig. II.8. The effect of phosphorus-starvation on the rate of uptake of ethanolaminephosphate-phosphorus by B. cereus (W). Cells were grown in PPYG medium as described in Materials and Methods. They were resuspended in the phosphorus-free medium (BXPG-NEM) and deprived of phosphorus. At various times, 0.5 mM [^{32}P]ethanolaminephosphate was added, and the rate of incorporation of ^{32}P by the cells followed. No period of deprivation, ●—● ; 2 h deprivation, ○—○ ; 4 h deprivation, ■—■ .

The Effect of Analogues on AEP Metabolism

Orthophosphite and four aminoalkylphosphonate derivatives were tested to see whether they had any effect on AEP metabolism. None of these compounds was able to act as a sole source of phosphorus, nor did any interfere with the growth of B. cereus when a source of phosphorus other than AEP was supplied (medium PPYG and PG). However, they all inhibited the growth of the organism, though to varying degrees, when AEP was the source of phosphorus (Table II.A).

The four phosphonates were then tested for their ability to act as "gratuitous inducers" of the AEP transport system. Aminomethylphosphonate was the most efficient of the four, and work was continued with this compound only. Phosphorus-starved cells, exposed to 0.5 mM aminomethylphosphonate for 2 h beforethe addition of AEP, took up AEP at almost maximal rates (Fig. II.9). The initial uptake (first 20 min) was not affected by either chloramphenicol (10 µg/ml) or actinomycin D (1 µg/ml) at concentrations which abolished induction of transport in cells starved in the usual manner (cf. Fig. II.2). Aminomethylphosphonate may be therefore considered to be a gratuitous inducer for the AEP transport system.

Table II.A. The effect of various compounds on the growth of *B. cereus* (W) in different media

The results are expressed as mg dry weight of cells per ml after 18 h growth. NG = no growth occurred.

Compound added (2.5 mM)	Medium PPYG	Medium PG	Medium AG	Medium BXPG
None	3.0	2.6	1.8	NG
Orthophosphite	2.8	2.2	NG	NG
Aminomethylphosphonate	2.9	2.4	0.01	NG
2-Amino-2-carboxyethylphosphonate	3.1	2.2	0.66	NG
1-Amino-n-propylphosphonate	3.0	2.4	1.4	NG
1-Amino-isopropylphosphonate	2.5	2.2	1.6	NG

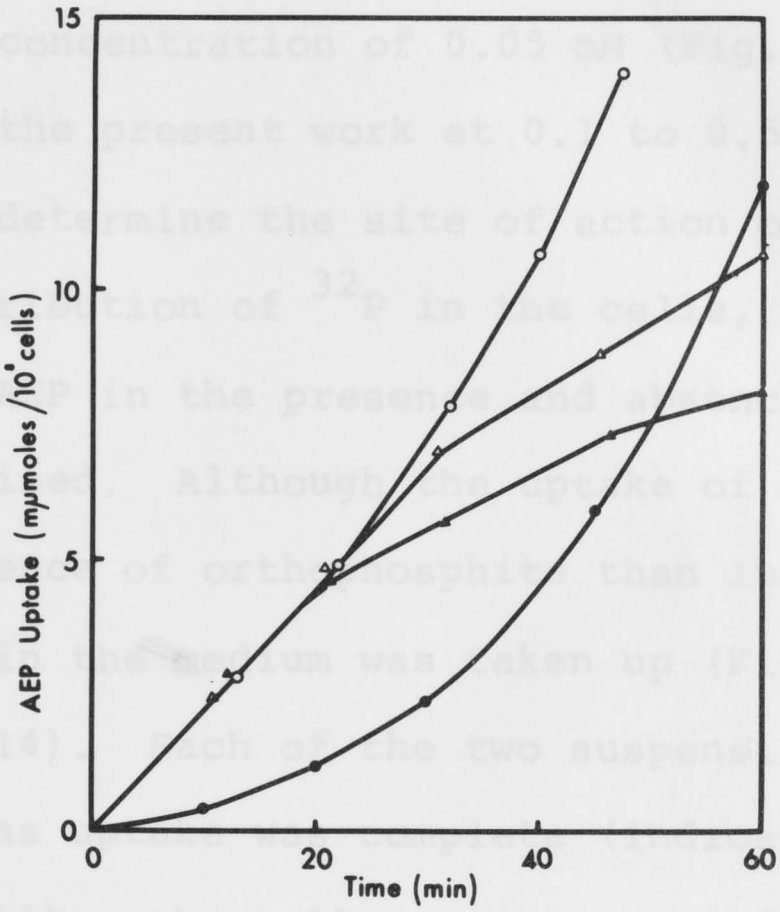


Fig. II.9. "Gratuitous" induction of AEP transport in B. cereus (W) by aminomethylphosphonate. Cells were prepared for uptake studies as described in Materials and Methods. They were then shaken for a further 2 h in the presence of 0.5 mM aminomethylphosphonate, after which they were washed free of the analogue and resuspended in BXPG-NEM containing 0.1 mM [^{32}P]AEP and the rate of uptake followed : Control (uninduced cells), ●——● ; pre-incubated cells, alone ○——○ , or in the presence of 10 μg/ml of chloramphenicol △——△ , or 1 μg/ml of actinomycin D ▲——▲ .

Further Investigation of the Inhibition Caused by Orthophosphite

Orthophosphite was found to inhibit growth at a minimal concentration of 0.05 mM (Fig. II.10), but was used in the present work at 0.1 to 0.5 mM.

To determine the site of action of orthophosphite, the distribution of ^{32}P in the cells, following the uptake of [^{32}P]AEP in the presence and absence of the inhibitor, was examined. Although the uptake of AEP was slower in the presence of orthophosphite than in its absence, all the AEP in the medium was taken up (Fig. II.11; cf. Fig. II.14). Each of the two suspensions was centrifuged as soon as uptake was complete (indicated by arrows, Fig. II.11); the pellets were suspended in 5 ml of 0.02 M acetic acid and heated on a boiling water bath for 5 min. The suspensions were centrifuged and the pellets re-extracted. The combined extracts were made to volume and the pellets suspended in a known volume of water. The radioactivity in the soluble, lipid and insoluble fractions was then determined. The bulk of the activity from the [^{32}P]AEP, taken up by the cells in the presence of orthophosphite, remained in the soluble fraction, while in the control suspension, about one-third or more of the radioactivity was incorporated into insoluble

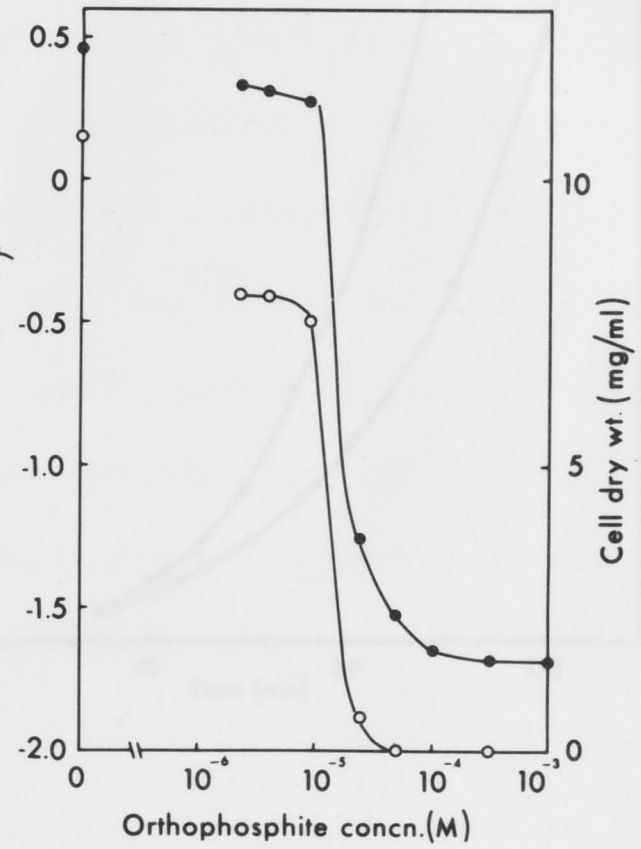


Fig. II.10. The inhibition by orthophosphite of the growth of B. cereus (W) on AEP as a sole source of phosphorus. Flasks containing AG medium and increasing concentrations of orthophosphite were inoculated and shaken for 17 h at 30°. Growth was assayed by measurement of $A_{660\ m\mu}$ (●——●) of appropriately diluted suspensions and as dry weight of a washed cell pellet (O——O).

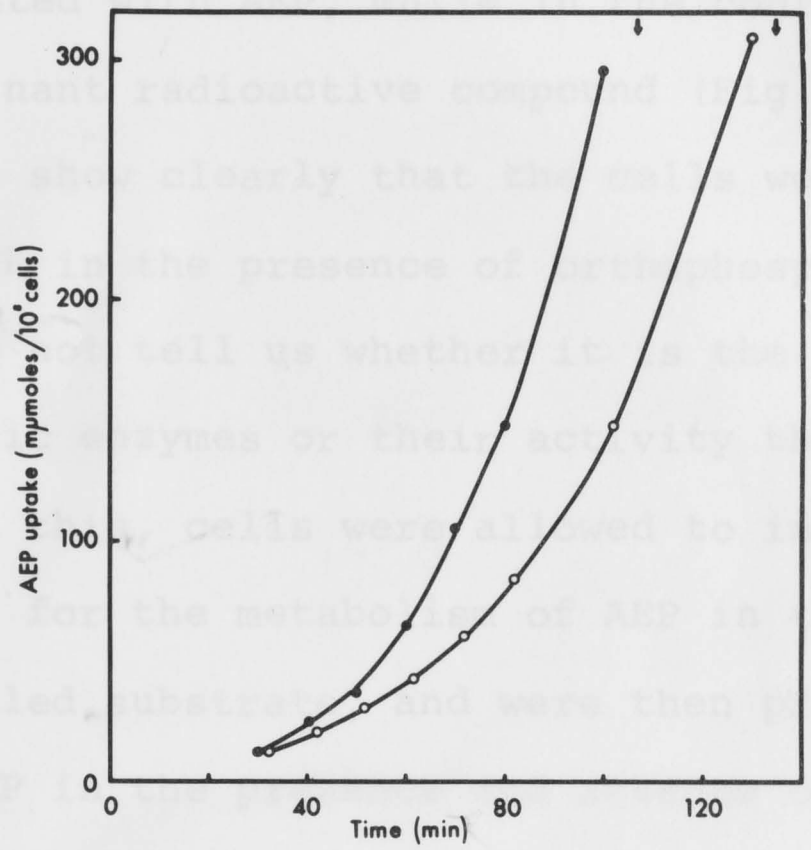


Fig. II.11. The uptake of AEP by B. cereus (W) in the presence and absence of orthophosphite. Phosphorus-starved cells, prepared as described in Materials and Methods, were resuspended in BXPG-NEM medium containing [^{32}P]AEP with (O——O) or without (●——●) 0.5 mM orthophosphite. The suspensions were sampled at various times to estimate the rate of uptake. The cells were harvested, at the times shown by arrows, when all of the AEP (300 mμmoles/10^9 cells) had been taken up.

components, including lipids. Electrophoretic examination of the soluble fractions showed that, in the presence of orthophosphite, most of the radioactivity was still associated with AEP, while in the control, Pi was the most predominant radioactive compound (Fig. II.12). These results show clearly that the cells were unable to break down AEP in the presence of orthophosphite; however, they do not tell us whether it is the formation of the catabolic enzymes or their activity that is inhibited. To test this, cells were allowed to induce the necessary systems for the metabolism of AEP in the presence of unlabelled substrate, and were then presented with [^{32}P]AEP in the presence and absence of orthophosphite. The results (Table II.B) show that under conditions where all the systems for the breakdown of AEP are functional, orthophosphite inhibits the activity of one (or more) of the catalytic enzymes.

Kinetic Experiments

The effect of varying substrate concentrations on the rate of AEP uptake is shown in Fig. II.13. The organism has a high affinity for AEP with an apparent Michaelis constant of 0.9×10^{-7} M; the maximum velocity ranged from 0.45 to 0.48 mμmole/min per 10^8 cells.

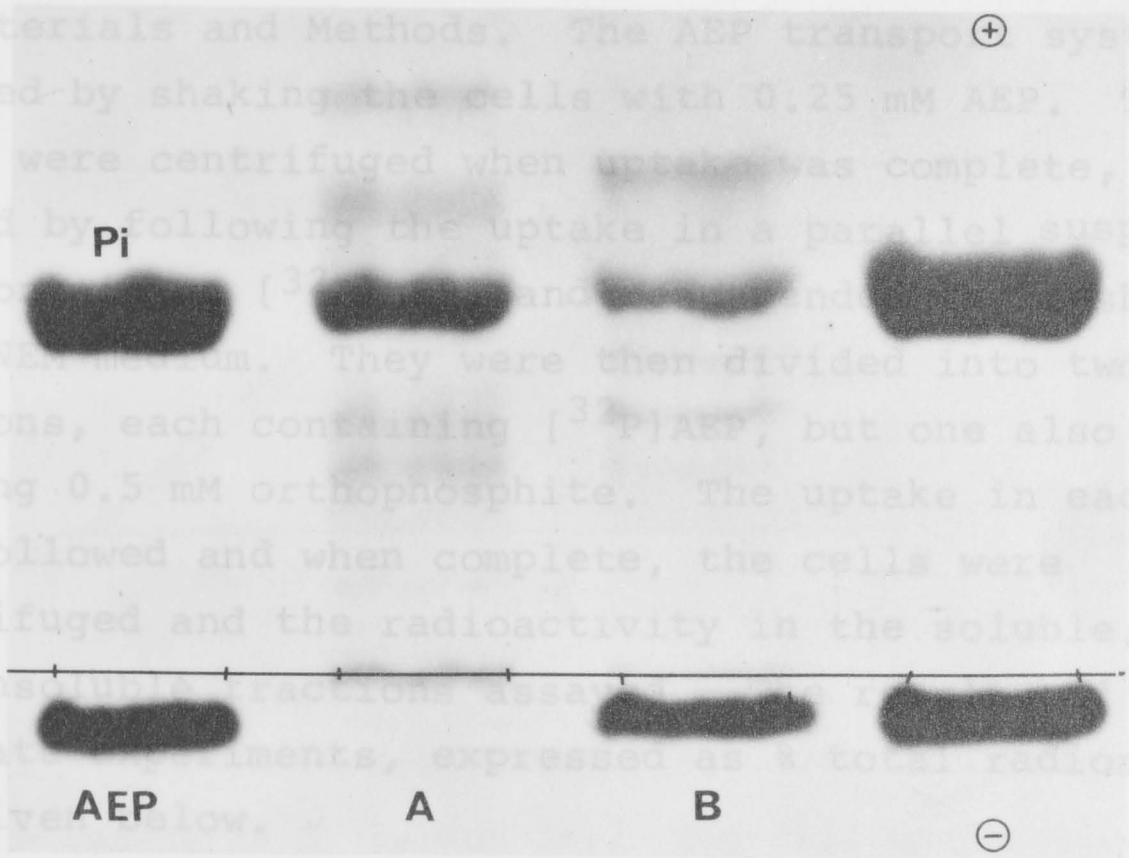


Fig. II.12. Autoradiograph of an electropherogram of the acid-soluble extracts of B. cereus (W) after the uptake of [^{32}P]AEP in the presence or absence of orthophosphite. The cell suspensions are those described in Fig. II.11.

A : cell extract (control)

B : as A, but with 0.5 mM orthophosphite present.

Table II.B. The effect of 0.5 mM orthophosphite on the distribution of radioactivity from [^{32}P]AEP in the soluble, lipid and residual fractions of *B. cereus* (W)

Phosphorus-starved cells were prepared as described in Materials and Methods. The AEP transport system was induced by shaking the cells with 0.25 mM AEP. The cells were centrifuged when uptake was complete, as judged by following the uptake in a parallel suspension containing [^{32}P]AEP, and resuspended in fresh BXPG-NEM medium. They were then divided into two portions, each containing [^{32}P]AEP, but one also containing 0.5 mM orthophosphite. The uptake in each flask was followed and when complete, the cells were centrifuged and the radioactivity in the soluble, lipid and insoluble fractions assayed. The results of two separate experiments, expressed as % total radioactivity, are given below.

Fraction	Per cent total radioactivity			
	Control		+ Orthophosphite	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Acid-soluble	82.0	74.0	97.9	93.5
Lipid	8.4	5.0	1.4	2.1
Residue	9.6	21.0	0.7	4.4

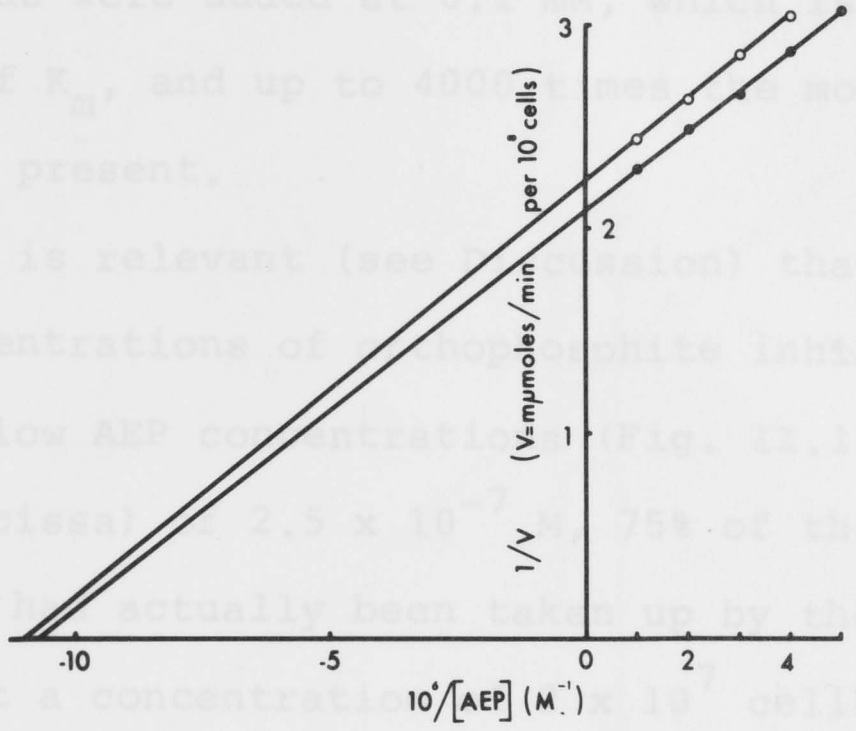


Fig. II.13. The effect of substrate concentration on the rate of AEP uptake in B. cereus (W). Two cell preparations were used (shown as ●——● and ○——○). The cells were pre-induced for AEP uptake by first starving in BXPG-NEM medium for 2 h at 10^9 cells per ml, followed by 2 h in the same medium in the presence of 0.5 mM unlabelled AEP. The cells were then washed free of AEP and diluted with BXPG-NEM medium to 2 x 10^7 cells per ml. The suspensions were cooled on ice and portions were withdrawn as required, pre-warmed to 30° and transferred to pre-warmed flasks containing the required amount of [^{32}P]AEP. Four uptake readings were taken for each concentration to ensure linearity of rates.

Neither Pi, nor orthophosphite had any effect on the apparent Michaelis constant for the uptake of AEP, nor on the value of V_{max} (Fig. II.14), although these compounds were added at 0.1 mM, which is 1000 times the value of K_m, and up to 4000 times the molecular ratio of the AEP present.

It is relevant (see Discussion) that in the presence of concentrations of orthophosphite inhibitory to growth, and at low AEP concentrations (Fig. II.14, last point on the abscissa) of 2.5×10^{-7} M, 75% of the substrate present had actually been taken up by the cells in 2 min. At a concentration of 2×10^{7} cells per ml and a mean cell volume of 4 μ^3, this represents a concentration against a gradient of 4×10^{4}.

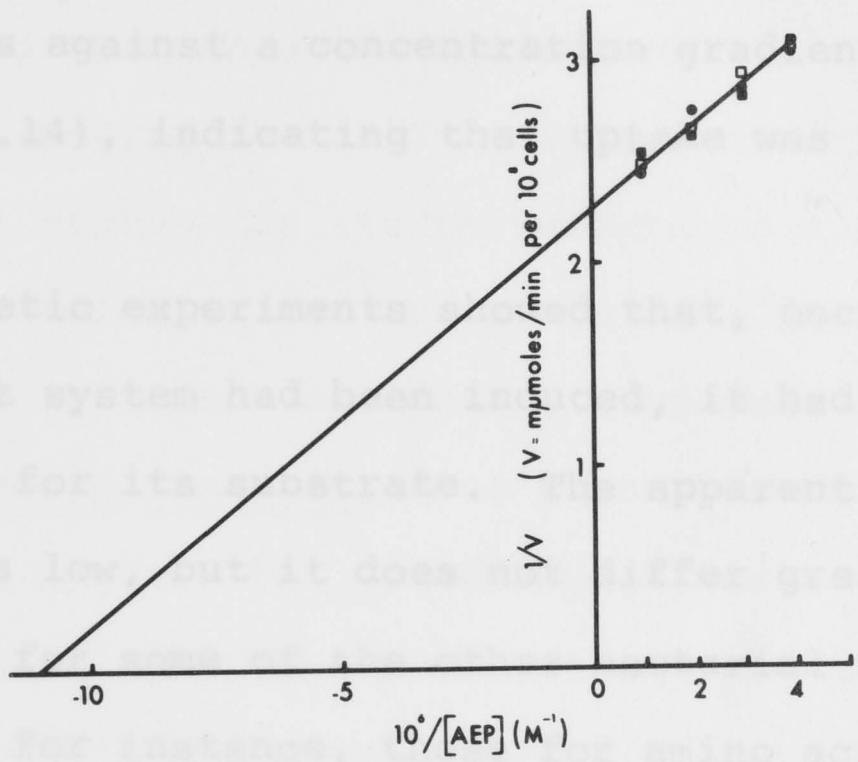


Fig. II.14. The effect of Pi and orthophosphite on the rate of AEP uptake in B. cereus (W). The reciprocal plots were obtained under conditions described in Fig. II.13. Control plot, ●——● ; as control, plus 0.1 mM Pi, ■——■ ; as control, plus 0.1 mM orthophosphite, □——□ .

DISCUSSION

The results presented in this chapter show that B. cereus has an inducible transport system for AEP. In the presence of concentrations of orthophosphite which abolished AEP breakdown, AEP was still accumulated by the cells against a concentration gradient of 4×10^4 (Fig. II.14), indicating that uptake was an active process.

Kinetic experiments showed that, once the AEP transport system had been induced, it had a high affinity for its substrate. The apparent K_m value of 10^{-7} M is low, but it does not differ greatly from those reported for some of the other bacterial transport systems, for instance, those for amino acids (in Escherichia coli, Britten and McClure, 1962; Piperno and Oxender, 1966, 1968; in Salmonella typhimurium, Ames, 1964), for sulphate (in S. typhimurium, Dreyfuss, 1964), for carbohydrates (in E. coli, Horecker, Thomas and Monod, 1960; in Staphylococcus aureus, Egan and Morse, 1966), and for Pi (in B. cereus, Rosenberg and La Nauze, 1968).

Although a number of workers have reported that bacteria can metabolize phosphonates (see Introduction), there has been only one other study of the transport of

phosphonates in microorganisms. Holden, van Balgooy and Kittredge (1968) found that Lactobacillus plantarum accumulated analogues of glutamic acid, aspartic acid, alanine and valine in which one of the carboxyl groups had been replaced by a phosphono group. Competitive studies with amino acids indicated that each phosphonic analogue entered the cell by way of the specific transport system for its corresponding amino acid. Kinetic studies of the transport of 2-amino-3-phosphono-propionic acid (the analogue of aspartic acid) in Streptococcus faecalis and a mutant lacking one of two transport systems for dicarboxylic acids also supported this theory. AEP (the analogue of β-alanine) was not taken up by either organism, and none of the phosphonates which were transported were metabolized to a significant extent within the cells. We have not tested β-alanine as a competitive inhibitor for the AEP transport system in B. cereus, but it seems unlikely that AEP enters this organism by way of a system designed to transport β-alanine for two reasons : firstly, β-alanine does not occur commonly in nature, and secondly, the severe repression exerted by Pi on the formation of the AEP transport system (Fig. II.6; also see Figs. II.1 and II.5) indicates that the function of this system is to

acquire phosphorus. Presumably, the ability to derive phosphorus from AEP is an advantage under certain conditions; however, the existence of a means for preventing AEP entering the cell when Pi is available, also indicates that it is metabolically costly for the cell to use AEP as a source of phosphorus.

Preliminary investigations with ethanolaminephosphate (EP) show that, even though it is structurally similar to AEP, it is assimilated by a different process. The rate of uptake of label from [^{32}P]EP was related to the state of phosphorus-deprivation of the cells, but the capacity to take up label formed in the absence of inducer (Fig. II.8). We have not examined the cells to see whether EP itself enters the intracellular space, or whether it is cleaved to Pi during transport by an alkaline phosphatase located in the cell membrane, or even by an extracellular form of this enzyme. The latter possibility may very well occur, since Cashel and Freese (1964) have found that, following phosphorus-starvation, Bacillus subtilis excretes alkaline phosphatase into the surrounding medium. We have found that B. cereus (W) does form alkaline phosphatase when it is deprived of Pi (see Chapters III and V), but we have not determined its location.

The inducible transport system for AEP described here displays many of the properties characteristic of other inducible systems. Thus, the induction process requires energy and an essential amino acid. It is abolished by agents, such as actinomycin D and chloramphenicol, known to inhibit the synthesis of messenger RNA and protein, respectively. Both the substrate and a non-metabolizable analogue can induce the transport system for AEP, which is then no longer affected by either actinomycin D or chloramphenicol (Figs. II.2 and II.6). Thus, this system resembles the inducible system for the metabolism for β-galactosides in E. coli, and the classical model proposed for that (Jacob and Monod, 1961) can equally well explain the present observations.

We have found that Pi is the major breakdown product of AEP within whole cells (Fig. II.12). The inhibition by Pi of the formation of the AEP transport system thus represents another instance of catabolite repression (Magasanik, 1961), the most extensively studied example of which is the inhibition by glucose of the expression of the lac operon in E. coli (Moses and Prevost, 1966; Paigen, 1966; Tyler, Loomis and Magasanik, 1967; Palmer and Moses, 1967, 1968; Moses and Yudkin, 1968; Yudkin and Moses, 1969). It is

curious that, although the production of the AEP transport system is inhibited by external Pi (Fig. II.6), it is unaffected by large amounts of Pi present intracellularly during AEP breakdown (Fig. II.12). Thus, it is evident that only extracellular Pi can act as a repressor. Other workers have reported similar findings. Sercarz and Gorini (1964) produced evidence which suggested that, in E. coli, the formation of the transport system for arginine is repressed by exogenous, but not endogenous, arginine. Dietz and Heppel (1969; also see Heppel, 1969), who also used E. coli for their studies, found that the formation of the transport system for glucose-6-phosphate could be induced by low concentrations of this compound in the surrounding medium; however, intracellular glucose-6-phosphate, even at concentrations as high as 0.04 M, was unable to act as an inducer for the system. As yet, there is no indication why these compounds should act as repressors (or inducers) only when they are present extracellularly. If the proteins of the transport system are synthesized in the cell membrane, it is possible that they can reach the site of protein synthesis only from the outside of the membrane (see also Heppel, 1969). In B. cereus, the action of Pi is similar to that of actinomycin D and it is tempting

to suggest that Pi also prevents the formation of messenger RNA; but, since it is now believed that, during protein synthesis, transcription is normally coupled to translation (Stent, 1964; Das, Goldstein and Lowney, 1967; Kaempfer and Magasanik, 1967) experiments designed to separate these two processes would have to be performed before this could be determined.

It is quite possible that, in microorganisms capable of growing on AEP, the repression by Pi of AEP utilization is a widespread phenomenon. We have found that it occurs in at least three other species of bacteria (Rosenberg and La Nauze, unpublished observations), but more species of bacteria would have to be tested before this theory could be verified.

Finally, it is interesting to note that B. cereus produces an extracellular lecithinase which is a phospholipase c, since it hydrolyses lecithin to phosphorylcholine and a diglyceride (Chu, 1949). Since AEP commonly occurs as a constituent of lipids (see Chapter I and the review by Kittredge and Roberts, 1969), it is quite likely that AEP is released from these lipids by the action of this enzyme and so becomes available to the bacterial cell for metabolism. Indeed, Hori, Arakawa, Sugita and Itasaka (1968) have reported that the α-toxin

from Clostridium perfringens, which is also a phospholipase c, does hydrolyse ceramide-AEP to ceramide and AEP; however, there have been no reports yet that this organism or other anaerobic bacteria can metabolise AEP.

CHAPTER III

The Isolation and Characterization of Two Mutants of *Bacillus cereus* with Altered Aspects of their Metabolism of 2-Aminoethylphosphonate

INTRODUCTION

In Chapter II, I described the inhibition by orthophosphite of the growth of the wild strain (W) of Bacillus cereus on AEP as the sole source of phosphorus. Orthophosphite was found to act by interfering specifically with the breakdown of AEP within the cells. This phenomenon was utilized for the selection of mutants. Thus, one of the mutants (S7-I) described in this chapter was isolated because it was able to grow on AEP in the presence of orthophosphite; however, it had also lost the ability to use Pi as a source of phosphorus. Further work showed that this mutant transported Pi and orthophosphite at only a fraction of the rate found in the wild strain, and that it had also become partially "constitutive" for the AEP transport system. The second mutant (AI-2), described in this chapter, was derived from S7-I as a revertant able to use Pi as a source of phosphorus. When this mutant was presented with AEP, it transported the compound immediately and at a linear rate. The mutant AI-2 had thus also acquired the property of a "constitutive" AEP transport system.

MATERIALS AND METHODS

Chemicals

The sources of most of the chemicals used (including labelled compounds) have been described in Chapter II. N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG) was a product of K and K Laboratories Inc. [^{32}P]Orthophosphite and [^{74}As]arsenate were obtained from the Radiochemical Centre, Amersham. The orthophosphite was purified by ion exchange chromatography (Pollard, Rodgers, Rothwell and Nickless, 1962). The arsenate required no further purification.

Microorganism and Media

Details concerning the parent organism (*B. cereus*, W), the conditions for its growth and the composition of the media used have also been described in Chapter II. In summary, the media consisted of a complex medium (PPYG), and a defined, phosphorus-free medium (BXPG). The latter was supplemented with 2.5 mM Pi (medium PG), AEP (medium AG), or ethanolaminephosphate (medium EG). In some cases, 0.1 mM orthophosphite or 0.25 mM arsenate were added to the AG medium (medium Ø-AG and α-AG, respectively). For solid media, Difco agar was added at 2% (w/v).

Uptake Studies

The methods for preparing cells for uptake studies and for determining the rate of transport of labelled-substrate into the cells are given in Chapter II.

Production and Selection of Mutants

Initially, cell suspensions were treated with MNNG as described by Adelberg, Mandel and Chen (1965), and the washed cells plated on, or inoculated into, Ø-AG medium for the selection of mutants. In later experiments, spontaneous mutants were also selected.

RESULTS

Selection of Mutants

The specific inhibitory effect of orthophosphite on the utilization of AEP offered a possible means for the selection of mutants able to grow in its presence. Mutations were induced by treatment with the mutagen MNNG (see Materials and Methods). After exposure to the mutagen for various times (5-10 min), the cells were washed and inoculated into Ø-AG medium or plated onto Ø-AG agar. Colonies began to appear after 24 h[a]. The mutants were isolated as pure lines and only those which produced a colony size 2 mm in diameter or larger, after incubation overnight at 30°, were retained. These were each inoculated in a patch onto PPYG agar, grown for 6 h, and replica-plated (Lederberg and Lederberg, 1952) onto PG, Ø-AG and α-AG agar. The plates were inspected after incubation overnight. In most cases, mutants which were able to grow well on Ø-AG medium also grew well on α-AG medium, but showed no

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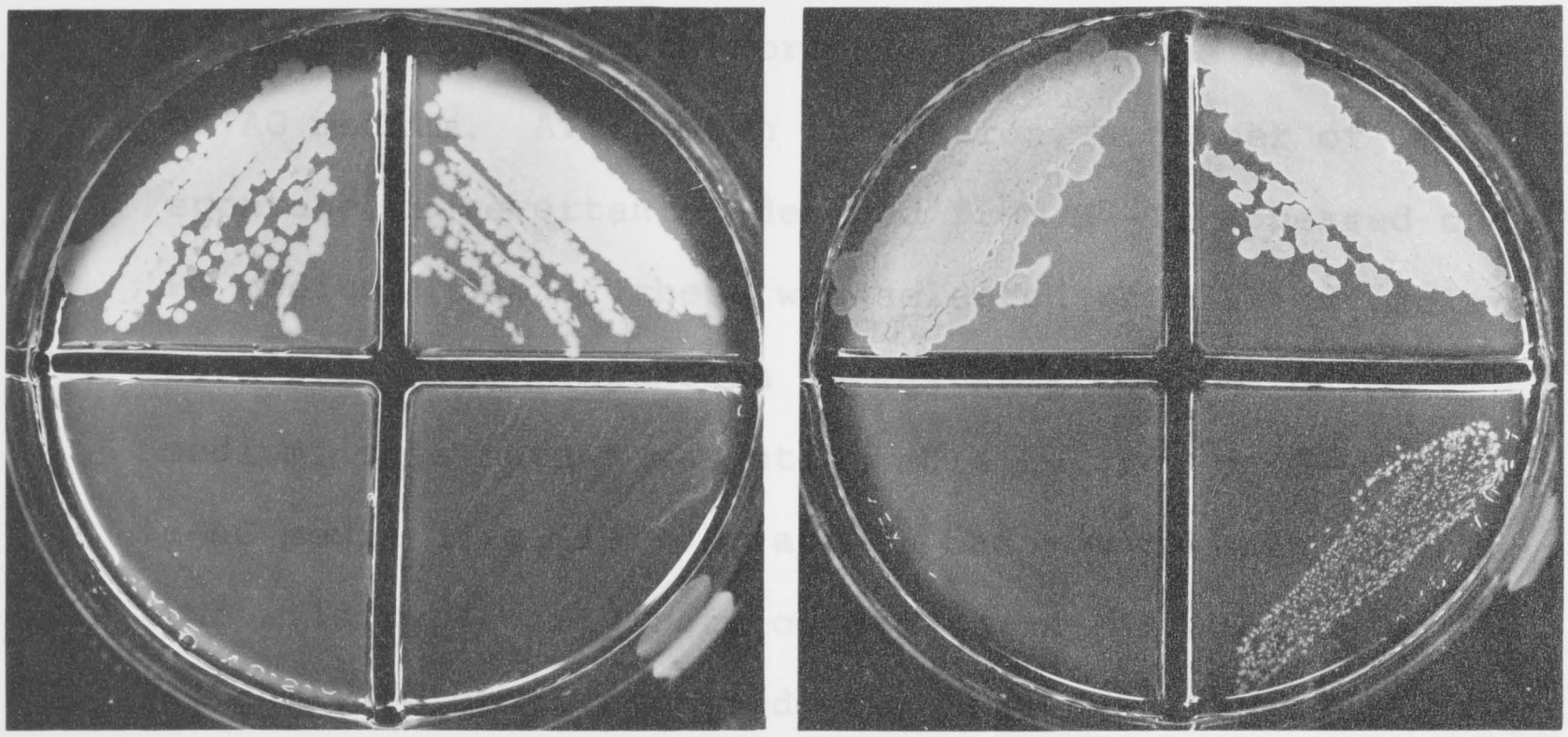
[a]Spontaneous mutants were also observed 24-48 h after untreated cells had been plated onto Ø-AG agar (see Fig. III.1). The fact that these were also true mutants was supported by good growth overnight when they were re-plated onto Ø-AG agar.



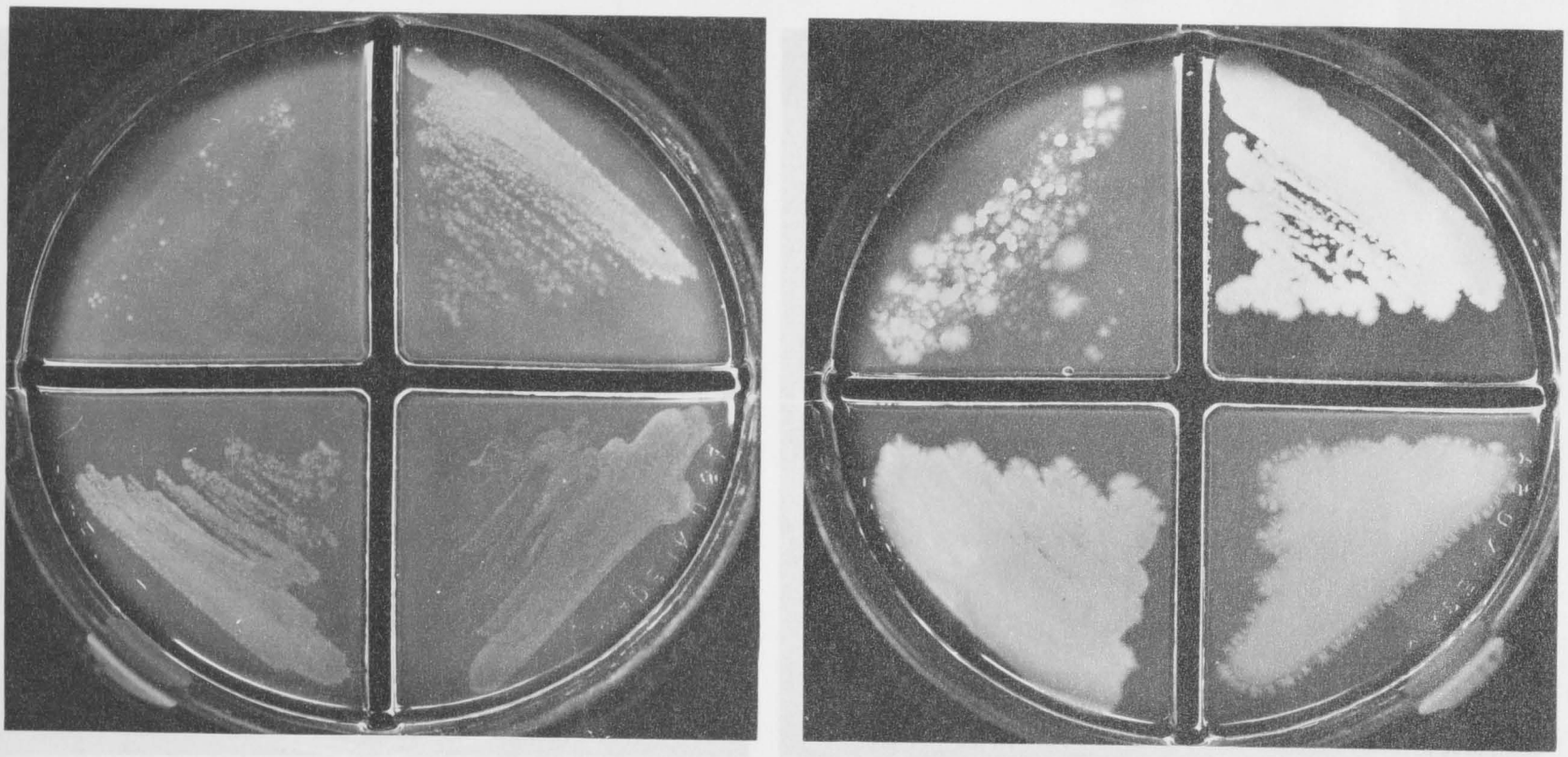
A B

Fig. III.1. The pattern of growth of the wild strain of B. cereus (W) on various media. Washed cells of B. cereus (W) were plated onto agar media and incubated for 24 h (plate A) and 48 h (plate B). The pattern of growth obtained is shown above. The media used were : top left sector, PG; top right sector, AG; bottom left sector, α-AG; bottom right sector, Ø-AG.

growth, or only poor growth, on PG medium. The growth pattern of the mutant finally selected (S7-I) is shown in Fig. III.2, and may be compared with that of the wild strain shown in the previous figure. The ability of this mutant to grow on Ø-AG and α-AG media is clearly evident; it does, however, grow more slowly than the wild strain on AG medium. After 48 h incubation, a number of spontaneous revertants, derived from S7-I, appeared on PG medium. One of these was selected (AI-2) and isolated as a pure line. Although it was able to grow on PG medium, this mutant was still able to grow on Ø-AG and α-AG media (Fig. III.3), and so had not reverted back to the wild type. Its rate of growth on Ø-AG medium is slightly retarded compared with that on other media, but is, nevertheless, faster than that of the mutant S7-I.

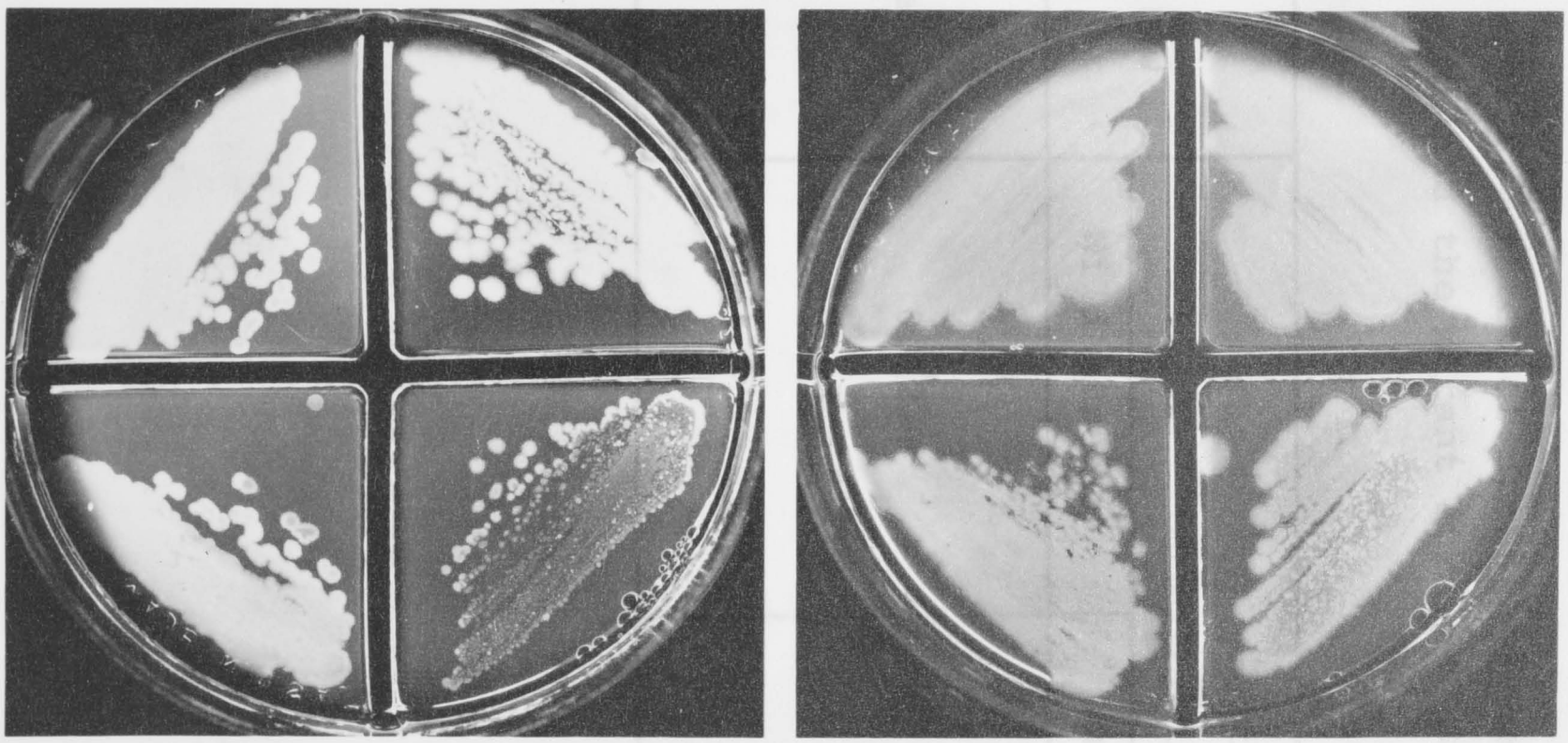
Uptake of Pi and Related Ions

The mutant S7-I took up Pi, orthophosphite, pyrophosphate and arsenate at only a fraction of the rate found in the wild strain (Table III.A). The simultaneous loss of the ability to take up all four anions suggested that these ions might share a common transport system. Further support for this hypothesis was gained from kinetic and counter-exchange studies with the wild strain (not reported here, see Rosenberg and La Nauze,



A B

Fig. III.2. The pattern of growth of the mutant strain (S7-I) of B. cereus on various media. Washed cells of B. cereus (S7-I) were plated onto agar media and incubated for 24 h (plate A) and 48 h (plate B). The pattern of growth obtained is shown above. The media used were : top left sector, PG; top right sector, AG; bottom left sector, α-AG; bottom right sector, Ø-AG.



A B

Fig. III.3. The pattern of growth of the mutant strain (AI-2) of B. cereus on various media. Washed cells of B. cereus (AI-2) were plated onto agar media and incubated for 24 h (plate A) and 48 h (plate B). The pattern of growth obtained is shown above. The media used were : top left sector, PG; top right sector, AG; bottom left sector, α-AG; bottom right sector, Ø-AG.

Table III.A. Initial rates of uptake of certain anions by the wild strain (W) and the mutant strains (S7-I and AI-2) of B. cereus

Anion (tested at 0.5 mM)	Uptake (mμmole/min/10^8 cells)		
	Wild strain	Mutant S7-I	Mutant AI-2
Pi	2.2	0.18	0.16
orthophosphite	0.12	0.015	0.019
pyrophosphate	0.43	0.026	-
arsenate	1.1	0.03	-

1968).

Although the mutant AI-2 was able to grow on PG medium, its rate of uptake of Pi and orthophosphite were comparable with the rates found in the mutant S7-I, and not those of the wild strain (Table III.A). The mutation which enables AI-2 to grow on PG medium is evidently not connected with the rate at which it transports Pi into the cell (see Discussion).

Further evidence that the metabolism of Pi by the two mutants is defective was obtained when colonies, grown on various media, were tested for their ability to hydrolyse p-nitrophenylphosphate (Table III.B). This test has been used to indicate the presence of alkaline phosphatase in Escherichia coli (Torriani and Rothman, 1961). The wild strain did not produce alkaline phosphatase when grown on Pi or PPYG media, but did so when other sources of phosphorus were used (phosphate-esters, or AEP). The two mutants, on the other hand, formed alkaline phosphatase under all conditions tested.

Uptake of AEP

The wild strain, under the standard conditions used for uptake studies, requires an induction period of about 40 min before it begins to transport AEP (see Chapter II). The rate of uptake of AEP by the mutant S7-I followed a

Table III.B. Alkaline phosphatase activity of strains of *B. cereus* grown on various media

The wild strain (W) and the mutant strains (S7-I and AI-2) of *B. cereus* were grown overnight at 30° on agar plates of different compositions shown below. The colonies were then sprayed with a solution of *p*-nitrophenylphosphate (10 mg/ml) in 0.1 M Tris-HCl buffer, pH 8.0 (Torriani and Rothman, 1961). Those colonies possessing alkaline phosphatase turned yellow within 1-2 min, and were scored as +.

Agar-medium	Wild strain	Mutant S7-I	Mutant AI-2
PPYG	-	+	+
PG	-	no growth	+
AG	+	+	+
EG	+	+	+

similar pattern, but without the lag period; cells presented with AEP began to transport it immediately, the rate of uptake increasing with time (Fig. III.4). The rate of uptake was not altered when either 0.5 mM Pi or arsenate was added; the presence of either of these two compounds prevented the formation of the transport system in the wild strain. Neither chloramphenicol nor actinomycin D affected the rate of uptake during the first 15-20 min (Fig. III.5). As shown in Chapter II, chloramphenicol and actinomycin D prevent the formation of the transport system in the wild strain.

The mutant AI-2, on the other hand, took up AEP at a linear rate immediately it was exposed to the compound. However, the rate was dependent on the state of phosphorus-deprivation of the cells (Fig. III.6), the rate reaching a maximum after 2-3 h starvation. At 2 h (the standard time of phosphorus-deprivation used for uptake studies), the rate was not altered by the presence of 0.5 mM Pi or arsenate (Fig. III.7). The addition of chloramphenicol or actinomycin D did not alter the initial rate of uptake. After 10 min, however, the chloramphenicol-treated cells started to transport AEP at a slower rate, and at 15 min, the actinomycin D-treated cells suddenly

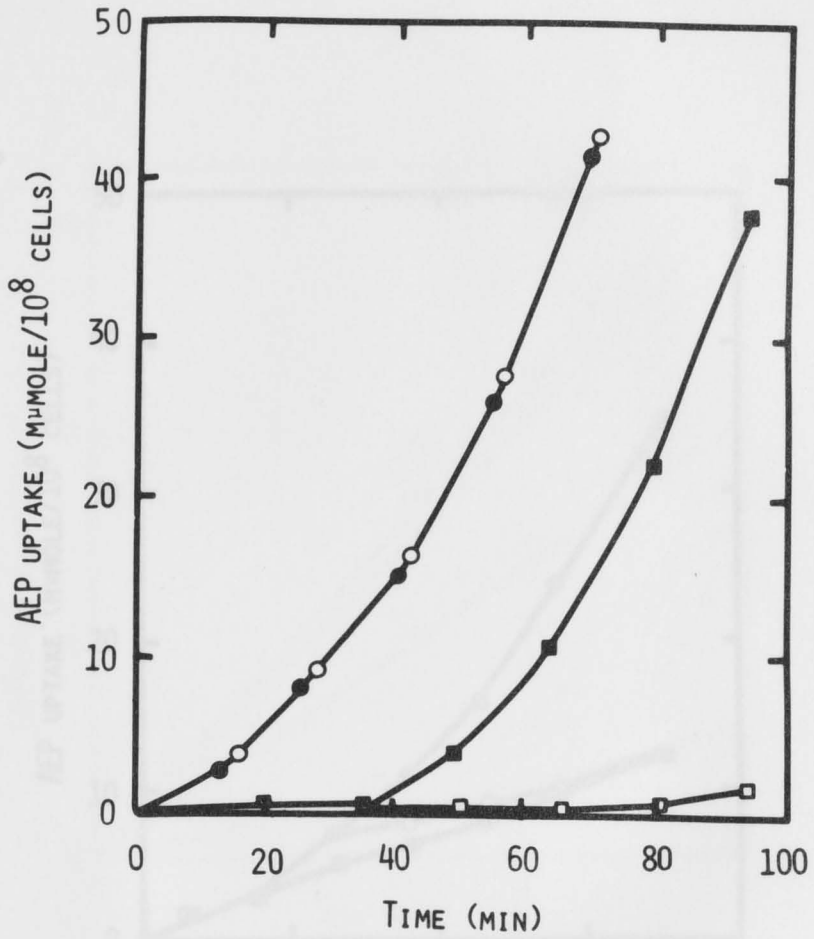


Fig. III.4. The rate of uptake of AEP in the mutant strain (S7-I) of B. cereus. Cells were prepared for uptake studies as described in Materials and Methods, Chapter II, and the rate of uptake of [^{32}P]AEP followed : no additions, ●——● , and with 0.5 mM Pi or arsenate added, O——O . For comparison, the uptake of AEP by the wild strain (W) under similar conditions is also shown : no additions, ■——■ , and with 0.5 mM Pi or arsenate added, □——□ .

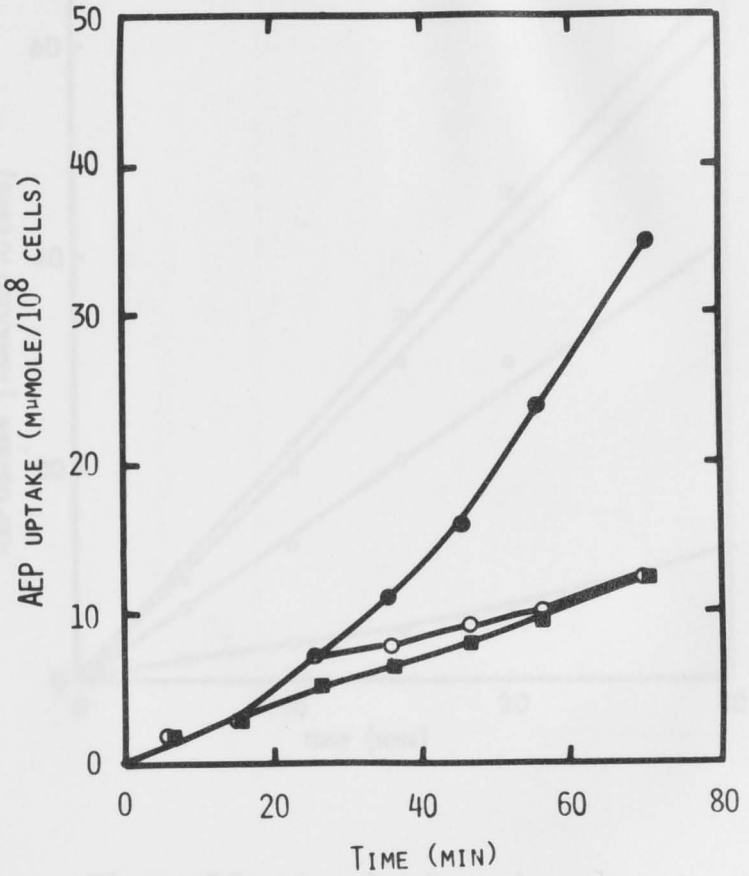


Fig. III.5. The effect of inhibitors of protein synthesis on the rate of uptake of AEP by the mutant strain (S7-I) of B. cereus. Cells were prepared for uptake studies as described in Materials and Methods, Chapter II. The rate of uptake of [^{32}P]AEP by the cells was followed : no inhibitor present (control), ●—● ; chloramphenicol (10 μg/ml), ○—○ , or actinomycin D (1 μg/ml), ■—■ , present.

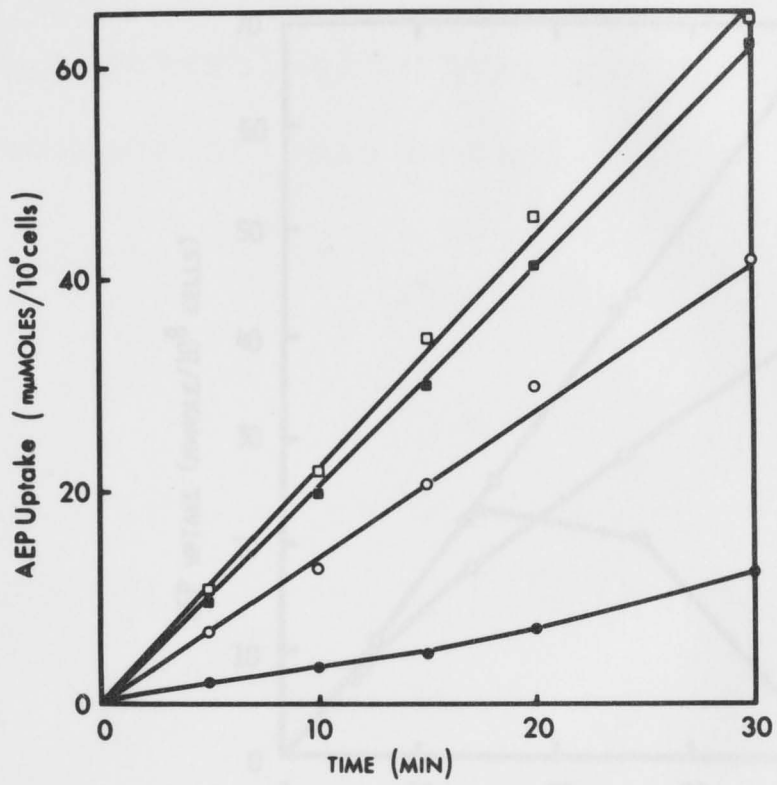


Fig. III.6. The effect of phosphorus-starvation on the rate of uptake of AEP by the mutant strain (AI-2) of B. cereus. Cells were grown in PPYG medium and resuspended in buffered, phosphorus-free medium (BXPG-NEM, see Materials and Methods, Chapter II). At various times, 0.5 mM [^{32}P]AEP was added, and the rate of uptake of the substrate by the cells followed. No phosphorus deprivation, ●—●; 1 h deprivation, ○—○; 2 h deprivation, ■—■; 3 h deprivation, □—□.

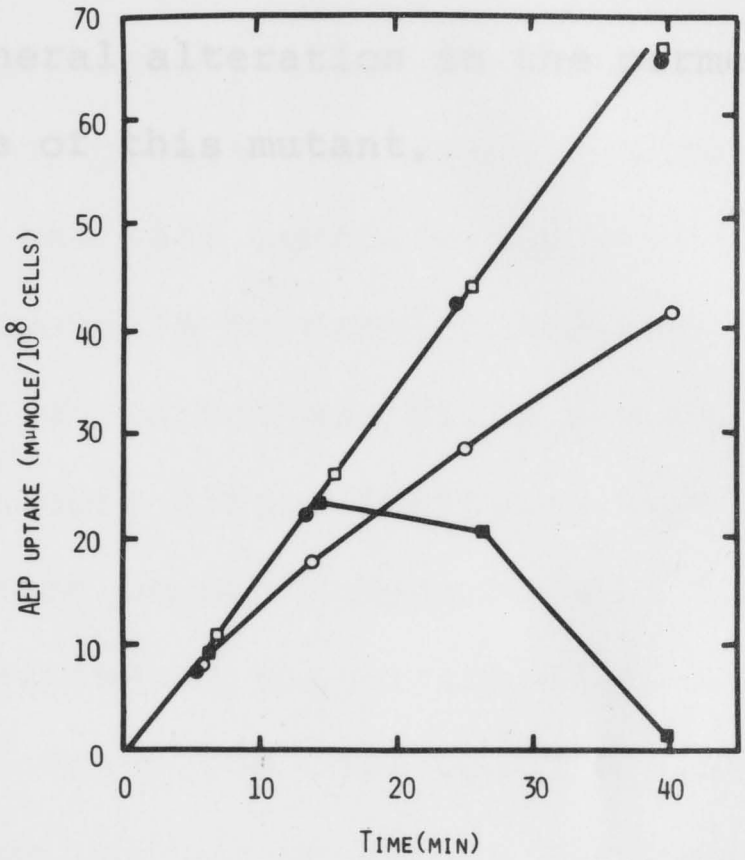


Fig. III.7. The rate of uptake of [^{32}P]AEP in the mutant strain (AI-2) of B. cereus. Cells were prepared for uptake studies as described in Materials and Methods, Chapter II, and the rate of uptake of [^{32}P]AEP followed : no additions, ●——● ; 0.5 mM Pi or arsenate added, □——□ ; chloramphenicol (50 μg/ml) added, ○——○ ; actinomycin D (2 μg/ml) added, ■——■ .

began to release the ^{32}P-label they had accumulated. The chemical nature of the radioactive material released has not been established. The reason why actinomycin D produces this effect in the mutant AI-2, and not in the wild strain or in the mutant S7-I, is not clear, but it may reflect a general alteration in the permeability of the cell membrane of this mutant.

DISCUSSION

In Chapter II, orthophosphite was shown to inhibit the utilization of AEP within the wild strain (W) of B. cereus. This specific effect has been used to select a mutant (S7-I) which was able to grow on AEP in the presence of orthophosphite. This mutant transported Pi, orthophosphite, pyrophosphate and arsenate at less than one-tenth of the rate found in the wild strain. The mutant was also able to grow on AEP in the presence of arsenate, but could not use Pi as a source of phosphorus. Such a pleiotropic effect indicated that all four anions might share some common component of a transport system which is defective in the mutant S7-I. The failure of these ions to enter the cell would explain why the mutant is able to grow on AEP in the presence of orthophosphite and arsenate, but fails to use Pi as a source of phosphorus. This mutant also had altered characteristics of its AEP transport system, as it did not exhibit a lag period (as the wild strain does) before induction took place. This could be because it is already deprived of endogenous phosphorus. Wu (1967) (also see Wu, Boos and Kalckar, 1969) has suggested that, in Escherichia coli, the functional integrity of the galactose transport system is necessary to retain pre-accumulated or intern-

ally generated galactose, and a similar situation could well exist in the mutant S7-I. Prolonged phosphorus-starvation (6 h) of the wild strain does result in a reduction of the time required for the induction of the AEP transport system; but, AEP must still be present for the system to be formed and it is not produced when either chloramphenicol or actinomycin D is also present (see Chapter II). In contrast, the addition of either chloramphenicol or actinomycin D does not alter the initial rate of uptake of AEP by the mutant S7-I, and this mutant may be considered to be partially "constitutive" for the AEP transport system. It seems likely that two separate mutations have occurred in S7-I, and it is possible that both are necessary for growth on AEP in the presence of orthophosphite. MNNG is a powerful mutagen and is capable of producing more than one mutation in each cell (Adelberg, Mandel and Chen, 1965).

The ability of AI-2 to use Pi as a source of phosphorus is puzzling, as it transports this ion at the same rate as S7-I and not at that of the wild strain. The mutant S7-I grows more slowly than either the wild strain or AI-2 on AEP (on which all can grow), suggesting that some other aspect of the metabolism of this organism is defective. It is possible that the mutation (or

reversion) which has occurred in AI-2 is one which enables it to retain intracellular Pi (see above). Alternatively, the utilization of Pi may be defective in S7-I; two intracellular pools of Pi are thought to exist in B. cereus (Rosenberg, Medveczky and La Nauze, 1969), and it may be that Pi slowly enters the first pool in both mutants, but only AI-2 is able to utilize Pi from this pool. Presumably, the concentrations of orthophosphite and arsenate entering the cells are not high enough to interfere with the overall metabolism of the organism, or else these ions are not transported from the first pool.

The mutant AI-2 was isolated from a medium containing Pi as the source of phosphorus, yet its pattern of uptake of AEP had also, unexpectedly, changed; it transported AEP at a linear rate. Although it is evident that the rate of uptake of AEP by AI-2 is influenced by the state of phosphorus-deprivation of the cells, this mutant has the ability to form the transport system for AEP in the absence of this compound acting as an inducer. Thus, compared with the wild strain, the mutant AI-2 may be considered to form the AEP transport system "constitutively".

A detailed genetic analysis of the number and

functions of the genes involved in the metabolism of Pi and AEP in B. cereus would help us to understand the behaviour of the mutants described above. Unfortunately, this is not practicable and outside the main theme of this work. There is no evidence at the moment that conjugation occurs in B. cereus. An earlier report by Felkner and Wyss (1964) of transformation in B. cereus 569 has since proved to be erroneous, as the organism was later shown to be B. subtilis (Goldberg and Gwinn, 1968). The isolation, recently, of a phage which carries out generalized transduction in B. cereus 569 (Thorne, 1968) is perhaps the first example of genetic exchange occurring in this organism. At this stage, a genetic approach to the problem seems formidable, especially when compared with the ease with which genetic studies can be carried out in E. coli K12.

CHAPTER IV

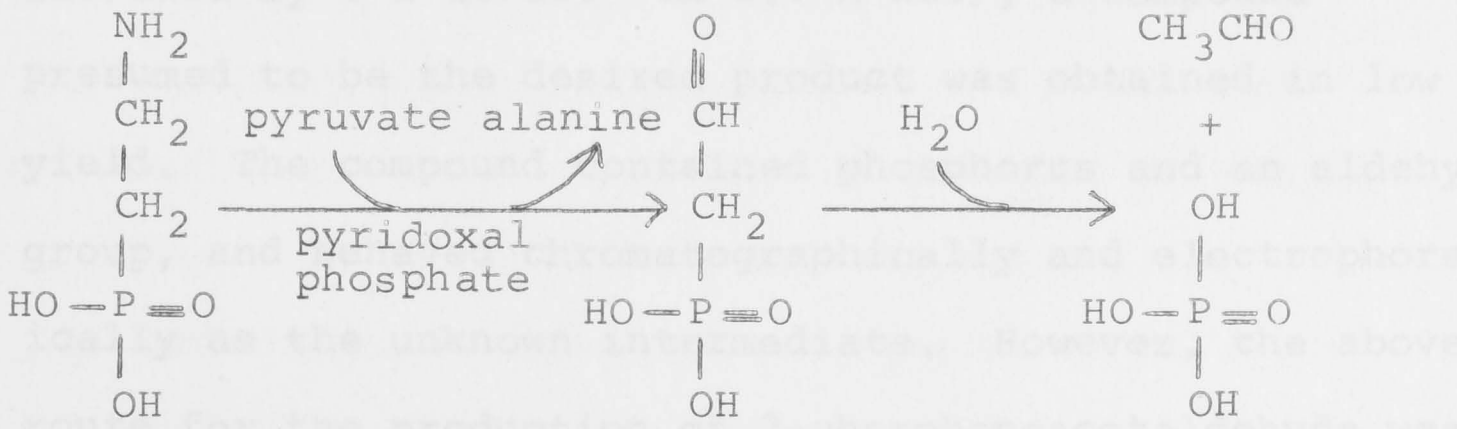
The Identification of 2-Phosphonoacetaldehyde as an Intermediate in the Degradation of 2-Aminoethylphosphonate by *Bacillus cereus*

INTRODUCTION

Although a number of workers in other laboratories have shown that bacteria are able to use phosphonates as sole sources of phosphorus (Zeleznick, Myers and Titchner, 1963; Mastalerz, Wieczorek and Kochman, 1965; Harkness, 1966), none described the cleavage of the carbon-phosphorus bond by cell-free preparations. However two hypothetical pathways by which microorganisms might degrade AEP have been put forward : Harkness (1966) proposed that, in Escherichia coli, a phosphate ester might be formed by a direct insertion of an oxygen atom between the carbon and phosphorus atoms, or by a phosphoryl group transfer reaction, and that a phosphatase was then required to liberate the Pi. De Koning (1966a) showed that, when AEP was heated with ninhydrin, Pi was released concomitantly with the development of ninhydrin colour, and on this basis, he suggested that 2-phosphonoacetaldehyde was formed by the deamination of AEP and that it decomposed spontaneously to acetaldehyde and Pi. Further, he suggested that this might be the means whereby the carbon-phosphorus bond was cleaved by living organisms.

In Chapter II, I showed that a strain of Bacillus cereus, isolated in this laboratory, was able to use

AEP as a sole source of phosphorus. The present chapter describes the preparation, from one of the mutants (AI-2) of B. cereus mentioned in Chapter III, of cell-free extracts which degrade AEP. During the course of AEP breakdown, two phosphorus-containing compounds (other than AEP) were found in the reaction mixture; one of these was Pi, but the identity of the other was not known. Preliminary results indicated that the enzymic breakdown of AEP proceeded by way of a transamination reaction, thus supporting the proposal of De Koning described earlier. The unknown compound, mentioned above, was isolated from the reaction mixture and identified as 2-phosphonoacetaldehyde by comparison on a number of criteria with the authentic material (Isbell, Englert and Rosenberg, 1969) thus establishing the following pathway for the degradation of AEP in B. cereus :



AEP

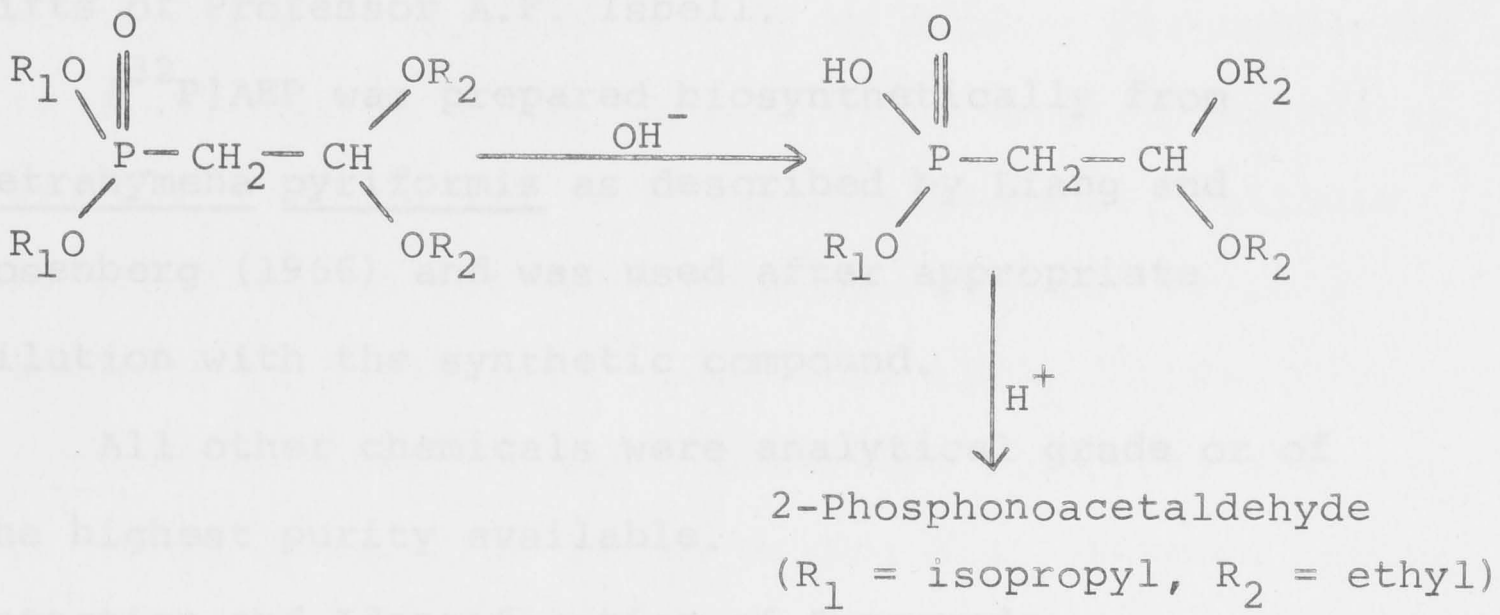
2-phosphono-acetaldehyde

acetaldehyde + Pi

MATERIALS AND METHODS

Chemicals

2-Phosphonoacetaldehyde. In the initial stages of this work, attempts were made to produce this compound by the controlled hydrolysis of an available diester, diacetal (provided generously by Professor J.M. Swan, of Monash University), thus :



Under optimal conditions (1 h at 100° in 1 M NaOH followed by 4 h at 100° in 2.5 M HCl), a compound presumed to be the desired product was obtained in low yield. The compound contained phosphorus and an aldehyde group, and behaved chromatographically and electrophoretically as the unknown intermediate. However, the above route for the production of 2-phosphonoacetaldehyde was abandoned following its successful synthesis, in good

yield and high purity, by the aqueous hydrolysis of 2-acetoxy-2-chlorethylphosphonyldichloride (Isbell, Englert and Rosenberg, 1969). The latter compound, a gift from Professor A.F. Isbell, was hydrolysed in water as required, and the 2-phosphonoacetaldehyde was purified as described below for the natural product.

The 2,4-dinitrophenylhydrazone of authentic 2-phosphonoacetaldehyde and crystalline AEP were also gifts of Professor A.F. Isbell.

[^{32}P]AEP was prepared biosynthetically from Tetrahymena pyriformis as described by Liang and Rosenberg (1966) and was used after appropriate dilution with the synthetic compound.

All other chemicals were analytical grade or of the highest purity available.

Detection and Identification of Compounds

Details of electrophoresis and the preparation of radioautographs have been described in Chapter II.

Ascending paper chromatography of 2,4-dinitrophenylhydrazone derivatives was carried out on Whatman No. 3 paper in the following solvent systems :

(1) Triethylamine-ether-pyridine-water (6:4:2:2, by vol.; Spare and Virtanen, 1961).

(2) n-butanol saturated with 0.5 M ammonia (Spare and Virtanen, 1961).

(3) sec-butanol-water (8:2, by vol.; Smith, 1967).

(4) n-butanol-ethanol-water (5:1:4, by vol.; Spare and Virtanen, 1961).

(5) N,N-dimethylformamide-ethanol-water (4:4:2, by vol.).

(6) methylcellosolve-ethanol-water (4:2:4, by vol.).

Compounds containing phosphorus were located on chromatograms as described by Rosenberg (1959). Aldehydes were detected either by dipping chromatograms in a solution of 0.01% (w/v) 2,4-dinitrophenylhydrazine and 0.2 M HCl in 90% (v/v) aqueous methanol, followed by 1.0 M KOH in 90% (v/v) aqueous methanol, or in a solution of 0.05% (w/v) ammoniacal $AgNO_3$ in 90% (v/v) aqueous acetone. In both cases, the aldehydes appeared as brown spots. The latter method also detected orthophosphite. The same reagents were used to locate, by spot tests on paper, the presence of compounds, containing either phosphorus or an aldehyde group, respectively in the effluents from columns.

AEP was detected on paper by dipping the chromatograms in a solution of 0.2% (w/v) ninhydrin in acetone and then heating them in an oven at 80° for 30 min. If this test is performed first, it is then possible to use the phosphorus reagent mentioned above on the same

chromatogram.

Radioactivity was measured using a Nuclear Chicago gas-flow counter fitted with a thin Mylar window.

Infrared spectra were determined with a Perkin-Elmer 621 dual grating spectrophotometer. Approximately 10% (w/v) solutions of the samples in ^2H_2O were examined as thin films between TlBr-TlI plates over the range 4000-200 cm^{-1}. There were two TlBr-TlI plates in the reference beam, but no ^2H_2O.

Proton magnetic resonance spectra were measured at 60 Mcycles/sec and 33.5° on a Perkin-Elmer R10 spectrometer. Approximately 10-20% (w/v) solutions of the samples in ^2H_2O were examined, and trimethylsilyl-propane sulphonic acid (sodium salt) was used as an internal indicator.

Preparation and Purification of 2,4-dinitrophenylhydrazones

Preliminary tests with authentic 2-phosphono-acetaldehyde showed that, while ethyl acetate extracted both the hydrazone and the reagent (2,4-dinitrophenyl-hydrazine) from 2 M HCl solutions, xylol extracted the reagent alone. The following procedure was therefore adopted : a sample of the isolated material was warmed to 60° in 2 M HCl containing 0.1% (w/v) 2,4-dinitro-

phenylhydrazine, and allowed to stand for 30 min. No crystallization occurred and the solution was extracted several times with xylol. The remaining aqueous solution was extracted with ethyl acetate and the extract was dried over Na_2SO_4 and filtered. The filtrate was taken to dryness and the material was dissolved in a small volume of ethyl acetate and applied to a column (92.5 x 25 cm) of cellulose powder (Whatman Standard Grade) prepared in a mixture of cyclohexane-ethyl acetate (4:1, by vol.). Using information derived from pilot runs on paper, the material was chromatographed by treating the column with mixtures of increasing proportions of ethyl acetate in cyclohexane, ethyl acetate alone and finally ethyl acetate containing increasing amounts of methanol. The fractions which emerged could be discerned by the yellow colour and that containing the desired compound was selected by paper chromatography in the presence of the reference compound.

Microorganism and Cell-Free Preparations

The wild strain (W) and the mutant strain (AI-2) of Bacillus cereus, used in the present work, have been described in Chapters II and III, respectively.

Details of the composition of the media used have

been given in Chapter II. The procedure adopted for the preparation of cell-free extracts from the mutant strain (AI-2) is described in Results.

Enzyme Assays

Enzyme assays were carried out at 25°. The details of the composition of the reaction mixtures are given in Results. Protein was estimated by the Biuret method.

Enzymic activity was estimated as follows :

(1) When [^{32}P]AEP was the substrate, the ^{32}Pi released was estimated by the method of Sugino and Miyoshi (1964) modified as follows : Protein was removed from the reaction mixture with 0.75 M $HClO_4$. H_2O_2 was added to a concentration of 4 mM in order to oxidise the dithiothreitol used in the assay system, and the ^{32}Pi released was precipitated as the triethylamine-molybdate complex. Detergent ("Shell-Nonidet", 0.005% by vol.) was added to the reagent to prevent the precipitate "creeping" up the walls of the test tubes. The precipitate was filtered on to membrane filters and washed with one-tenth strength reagent. The dried membranes were fixed to planchettes and counted in a Nuclear Chicago gas-flow counter. [^{32}P]AEP did not interfere with this method.

(2) When 2-phosphonoacetaldehyde was the substrate, the protein was removed from the reaction mixture with 0.75 M $HClO_4$ or 5% (w/v) trichloroacetic acid, and the Pi released estimated by the method of Harris and Popat (1954).

The liberation of acetaldehyde as a reaction product was demonstrated as follows : 3 ml of 0.1% (w/v) solution of 2,4-dinitrophenylhydrazine in 2 M HCl was placed in the outer well of a Conway Diffusion Unit, and the reaction mixture, in a volume of 1 ml, was placed in the centre well. The unit was sealed and placed in an incubator at 30° for 2 h. Crystals of the resulting 2,4-dinitrophenylhydrazone precipitated in the outer well.

RESULTS

The Preparation of Cell-Free Extracts

Preliminary experiments showed that bisulphite inhibited the growth of B. cereus (W) to a greater degree when AEP was the source of phosphorus than when Pi was used (Fig. IV.1). This indicated that aldehydes might be involved in the degradation of AEP by this organism. Further support for this was obtained when bisulphite was shown to inhibit the degradation of [^{32}P]AEP by whole cells (Fig. IV.2). The mutant AI-2 was used for this experiment. It is "constitutive" for the AEP transport system; cells not previously exposed to AEP take it up immediately and at a linear rate (see Chapter III). It seemed possible that this organism might also be "constitutive" for the enzyme(s) required to break down AEP; the results of the experiment shown in Fig. IV.2 demonstrate that these enzymes are certainly active in whole cells of the mutant. I therefore decided to use it to study the catabolism of AEP in cell-free systems, especially since my attempts, at this stage, to prepare active extracts from the inducible strain (W) had been unsuccessful.

In Chapter III, it was shown that the uptake of

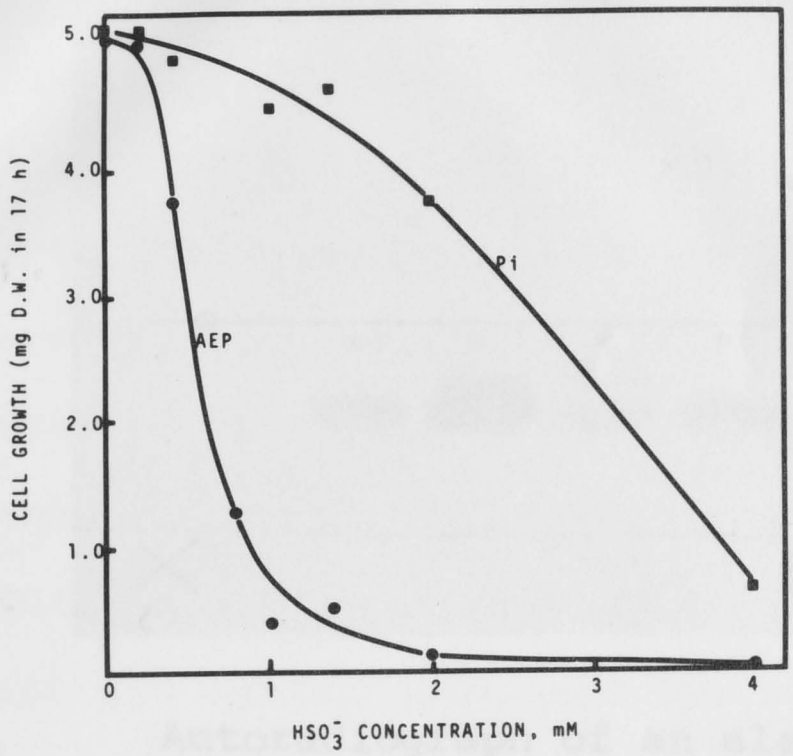


Fig. IV.1. The effect of bisulphite on the growth of B. cereus (W). Cells were inoculated into flasks containing BXPG medium with either Pi or AEP as the source of phosphorus (medium PG or AG, respectively) and a range of concentrations of $NaHSO_3$. After 17 h, the growth of the bacteria in each flask was estimated as mg dry weight/ml.

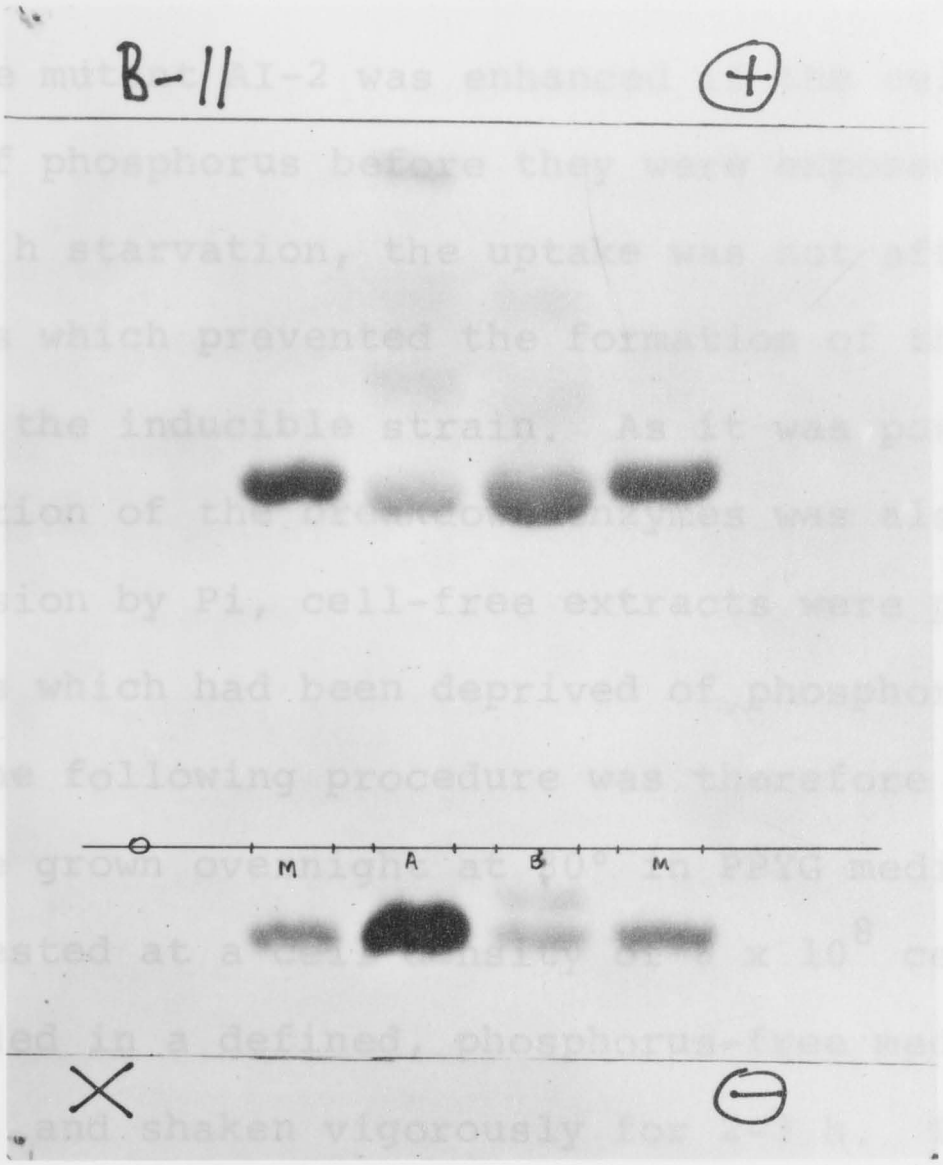


Fig. IV.2. Autoradiograph of an electropherogram of the acid-soluble extracts of B. cereus (AI-2) after the uptake of [^{32}P]AEP in the presence or absence of bisulphite. Cells were prepared for uptake studies as described in Materials and Methods, Chapter II. They were resuspended, at a cell density of 10^9 cells/ml, in fresh BXPG-NEM medium containing 0.1 mM $SO_4^=$ (i.e. one-third the normal concentration). The cell suspension was then added to two flasks, one of which contained 10 mM HSO_3^-. 0.5 mM [^{32}P]AEP was added to each and, when uptake was complete, the cells were removed by centrifugation. The pellet was resuspended in 2 ml of 0.02 M acetic acid and heated for 5 min in a boiling water bath. A sample of the acid-soluble extract was electrophoresed at pH 2 and a radioautograph prepared.

A : cell extract, with 10 mM $NaHSO_3$

B : cell extract, with no additions (control).

AEP by the mutant AI-2 was enhanced if the cells were starved of phosphorus before they were exposed to AEP. After 2-3 h starvation, the uptake was not affected by conditions which prevented the formation of the uptake system in the inducible strain. As it was possible that the formation of the breakdown enzymes was also subject to repression by Pi, cell-free extracts were prepared from cells which had been deprived of phosphorus for 2-3 h. The following procedure was therefore adopted : cells were grown overnight at 30° in PPYG medium. They were harvested at a cell density of 8×10^8 cells/ml, re-suspended in a defined, phosphorus-free medium (BXPG-NEM) and shaken vigorously for 2-3 h. The suspension was centrifuged, and the cells re-suspended in 4 vol. of cold, 50 mM N-ethylmorpholine-HCl buffer (pH 7.5) containing EDTA (10 mM) and dithiothreitol (1-5 mM)[a]. The cells were broken in a Sorvall Ribi Fractionator at 25,000 lb/inch^2, the homogenate

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[a]Saier and Jenkins (1967) reported that EDTA and dithiothreitol protected alanine aminotransferase from pig heart against loss of activity through aerial oxidation. These two compounds were therefore added to the buffer to protect the activity of the transaminase thought to be involved in the degradation of AEP to Pi.

centrifuged at 4° for 15 min at 38,000 x g, and the supernatant used for enzyme assays.

The reaction was followed for 1 h, over which time the rate of formation of Pi was linear. Table IV.A. shows the rate of liberation of Pi from AEP, by the cell-free preparation, under various conditions. The reaction rate was increased by the addition of pyridoxal phosphate, whereas pyridoxal was ineffective. The presence of the amino-group acceptor, pyruvate, was essential. Oxaloacetate could replace pyruvate to a certain extent, but this may have been a result of its decarboxylation to pyruvate by the crude extract used. The reaction was partly inhibited by isonicotinic acid hydrazide, and completely by hydroxylamine and bisulphite. The reaction was not affected by Pi (5 mM), but was strongly inhibited by orthophosphite, which interferes with the growth of B. cereus on AEP (see Chapter II).

Electrophoretic examination of the reaction mixture showed two radioactive products (Fig. IV.3). The major (slower moving) compound was identified as Pi with the phosphate reagent of Rosenberg (1959). The minor product gave a transient colour on paper with this reagent, a brown colour with 2,4-dinitrophenyl-hydrazine and a faint brown colour with ammoniacal

Table IV.A. Factors affecting the release of Pi from AEP by a cell-free preparation of B. cereus (AI-2)

The complete mixture contained : 2-amino-2-methyl-1,3-propandiol-HCl buffer (100 mM, pH 8.4); [^{32}P]AEP, specific radioactivity 10^4 counts/min per µmole, 4 mM; sodium pyruvate, 5 mM; potassium EDTA, 5 mM; dithiothreitol, 2.5 mM; pyridoxal phosphate, 0.5 mM; and the bacterial extract (5 mg protein per ml of reaction mixture). The mixture was incubated for 30 min at 25° and the reaction stopped by the addition of 0.75 M $HClO_4$. The Pi released from AEP was estimated as described in Materials and Methods.

Alterations to reaction mixture	Activity (mµmoles Pi released per h per mg protein)
None (complete system)	284
Omissions :	
Pyridoxal phosphate	94
Pyruvate	4
Pyridoxal phosphate and pyruvate	<2
Substitutions :	
Pyridoxal for pyridoxal phosphate	94
Oxaloacetate for pyruvate	123
α-Ketoglutarate for pyruvate	7
Additions (all at 5 mM) :	
Isonicotinic acid hydrazide	120
Hydroxylamine	<2
Bisulphite	<2
Orthophosphite[a]	42
Orthophosphate	286

[a]Reaction ceased after 20 min.

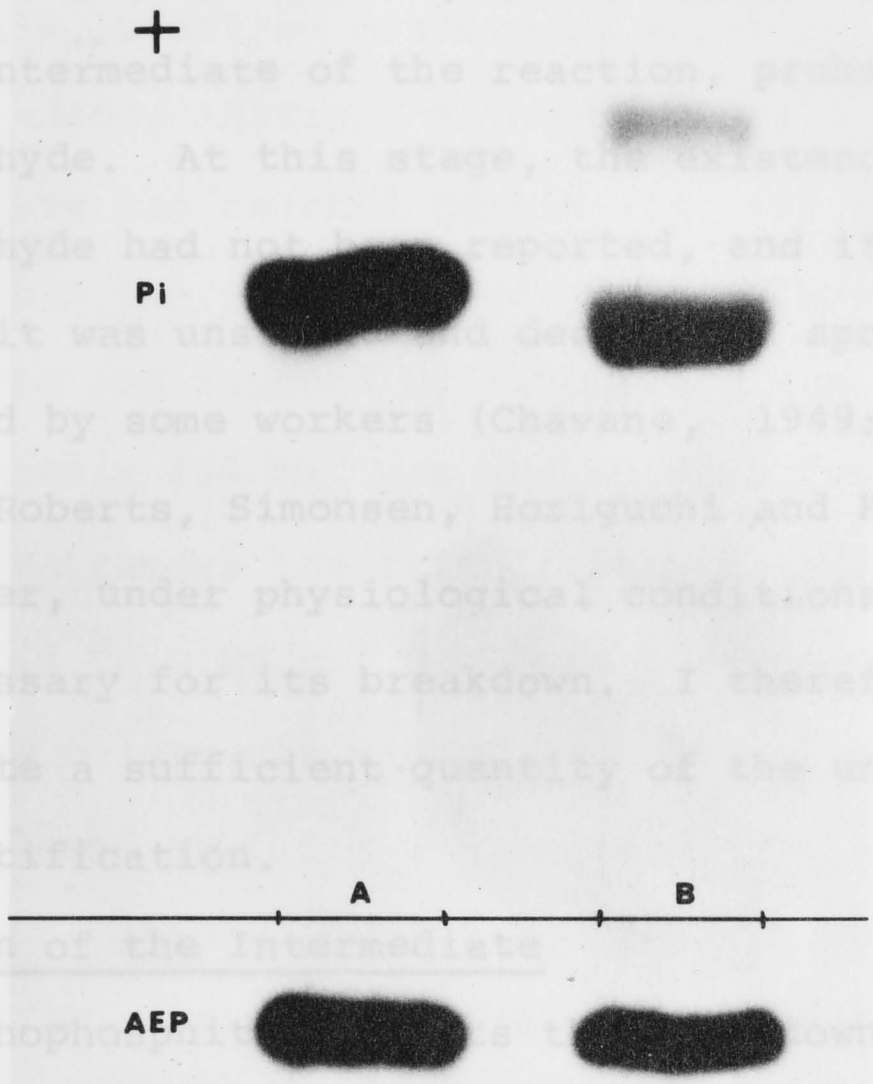

Fig. IV.3. The breakdown of [^{32}P]AEP by cell-free extracts of B. cereus (AI-2). The reaction products were electrophoresed at pH 2 and radioautographed as described in Materials and Methods. A, reference compounds; B, reaction mixture.

silver nitrate. This compound was always found in the complete reaction mixture, and it seemed likely that it was an intermediate of the reaction, probably 2-phosphonoacetaldehyde. At this stage, the existence of 2-phosphonoacetaldehyde had not been reported, and it was not known whether it was unstable and decomposed spontaneously as suggested by some workers (Chavane, 1949; De Koning, 1966a; Roberts, Simonsen, Horiguchi and Kittredge, 1968), or whether, under physiological conditions, an enzyme was necessary for its breakdown. I therefore attempted to isolate a sufficient quantity of the unknown compound for identification.

Isolation of the Intermediate

Orthophosphite inhibits the breakdown of AEP to Pi, both within whole cells (see Chapters II and III) and in cell-free systems (Table IV.A). Electrophoretic examination of a reaction mixture, similar to that in Table IV.A, showed that, although the release of Pi had been suppressed, the intermediate (presumed to be 2-phosphonoacetaldehyde) was still formed, and in relatively larger amounts. Therefore, in the work reported below, orthophosphite was added to the reaction mixture to accumulate larger quantities of this compound.

A large-scale reaction mixture was incubated with

[^{32}P]AEP in the presence of orthophosphite. The progress of the reaction was followed by sampling a parallel, small-scale, reaction mixture to which orthophosphite had not been added. After 2 h, when the release of ^{32}Pi in this mixture had reached completion, the protein in the main mixture was precipitated with 5% (w/v) trichloroacetic acid and removed by centrifugation. The supernatant (approximately 250 ml) was shaken with four 250-ml portions of ether to remove the trichloroacetic acid, and applied to a column (3 cm x 22 cm) of Dowex I X-8 (acetate) 100-200 mesh. The column was washed with 500 ml of distilled water followed by 0.1 M acetic acid. This procedure removed the cations and any [^{32}P]AEP which remained in the reaction mixture. The column was treated with 2 M HCl and the eluate taken to dryness under reduced pressure. The HCl was removed from the residue by repeated evaporation with water. The material was finally dissolved in water and the solution (pH 2) adjusted to pH 7 with 1 M NaOH before it was applied to a column (2 cm x 54 cm) of Dowex I X-8 (HCO_3^-) 100-200 mesh (Martonosi, 1960). The column was washed with water and eluted at 4° with a linear gradient (Snyder, 1965) of $KHCO_3$ ranging from 0-0.8 M; the effluent was collected, in 11-ml fractions, into tubes containing sufficient moist Dowex-50 (H^+) to react with

all the bicarbonate present[a]. The radioactivity emerged in three peaks (Fig. IV.4). Spot tests showed that the first and the third peaks contained a compound with a phosphorus and an aldehyde group, and that the second peak contained both Pi and orthophosphite. The fractions comprising each of these peaks were pooled and the solutions concentrated. Electrophoretic examination confirmed the presence in the third peak of a compound which migrated as the unknown aldehyde; this minor peak is probably caused by the slow equilibrium between the aldehyde and its enol form (cf. infrared and proton magnetic resonance studies). The concentrated solution from the first peak was treated with acid-washed charcoal (Norit-A), and reduced in volume to 0.4 ml before it was applied to a column of Sephadex G-10 (0.6 cm x 88 cm). The column was eluted with distilled water at 4°; the effluent was collected in 0.25-ml fractions. Two minor radioactive peaks emerged, followed by a third larger peak (Fig. IV.5). The minor peaks were shown, by electrophoresis and radioautography, to contain two radioactive compounds, both of which gave a

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[a]This procedure reduced the time spent by the solutes in the alkaline bicarbonate solution and resulted in minimal losses of the product.

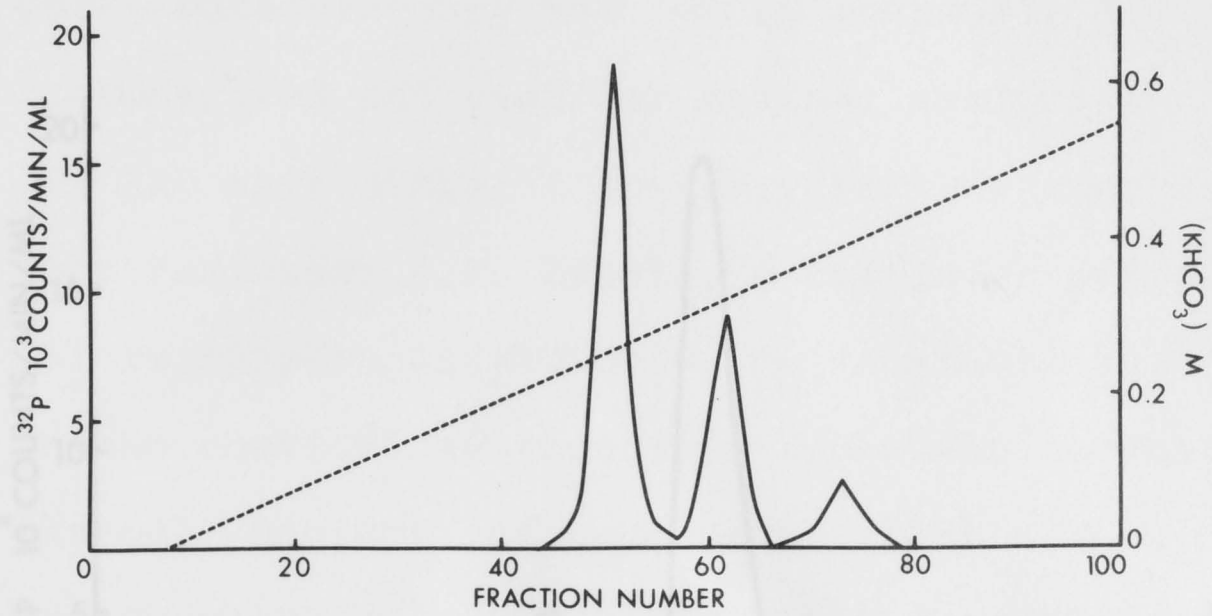


Fig. IV.4. Fraction on Dowex-1 (bicarbonate) of the breakdown products of [^{32}P]AEP. The large-scale reaction mixture contained, in addition to 1 mM orthophosphite, 1 mmole of AEP and the other components complete assay system as given in Table IV.A. After incubation for 2 h at 30°, the protein was removed and the extract subjected to a preliminary passage through Dowex-1 (acetate), as described in the text. The partially purified material was then applied to the Dowex-1 (bicarbonate) column, eluted with a linear gradient of $KHCO_3$ (- - - - -, theoretical gradient), and the radioactivity of the effluent monitored (———).

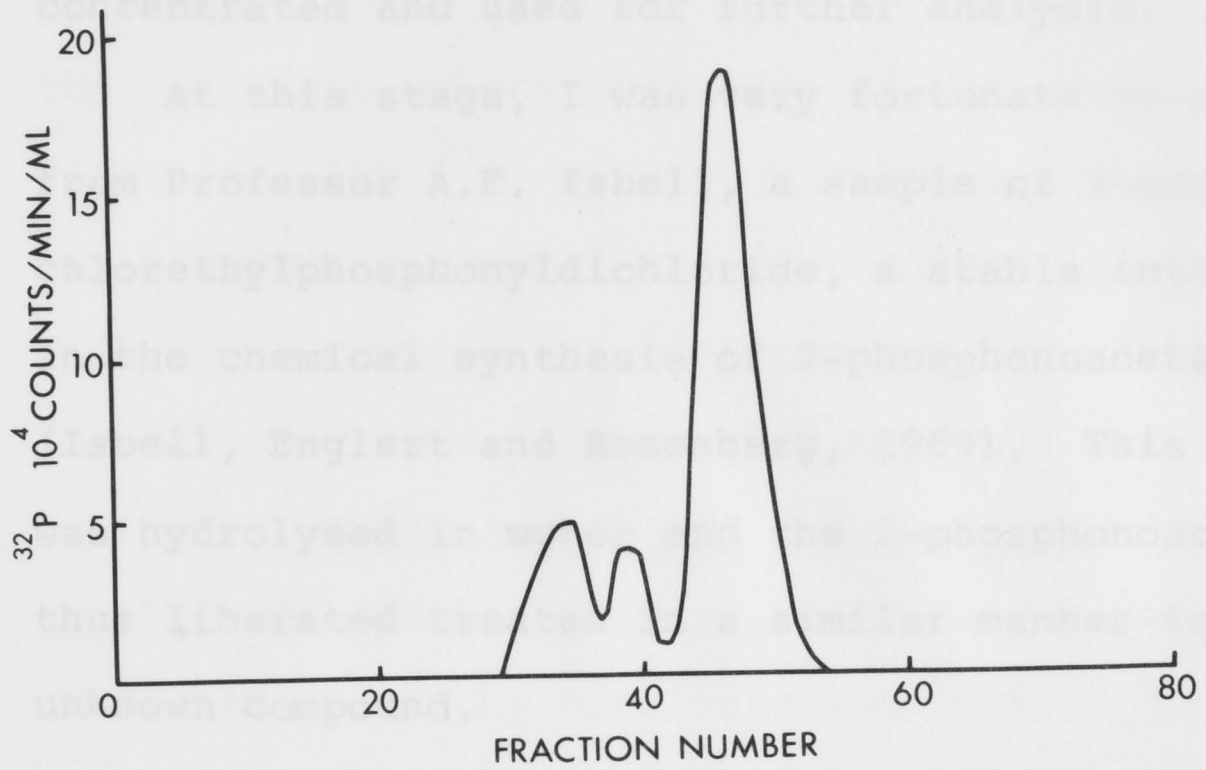


Fig. IV.5. Gel filtration on Sephadex G-10 of the concentrated material from the first peak, Fig. IV.4. The above curve shows the emergence of radioactivity on elution with water.

positive test on paper for an aldehyde group. One of these ran in a position which corresponded with the unknown aldehyde; the other compound ran ahead of it and was possibly a condensation product. The larger peak contained only the former compound, and was concentrated and used for further analysis.

At this stage, I was very fortunate to receive, from Professor A.F. Isbell, a sample of 2-acetoxy-2-chlorethylphosphonyldichloride, a stable intermediate in the chemical synthesis of 2-phosphonoacetaldehyde (Isbell, Englert and Rosenberg, 1969). This compound was hydrolysed in water and the 2-phosphonoacetaldehyde thus liberated treated in a similar manner to the unknown compound.

Comparison of the Isolated Material with Authentic 2-Phosphonoacetaldehyde

(1) Chromatography and electrophoresis. The two samples showed the same electrophoretic mobility at pH 2.0 (cf. Fig. IV.3). They also migrated identically in two chromatography systems : in water-saturated phenol and in acetone-acetic acid-water (5:3:2, by vol.), the R_f values were 0.44 and 0.62, respectively.

(2) 2,4-Dinitrophenylhydrazones. The purified 2,4-dinitrophenylhydrazone of the natural material was compared with the authentic derivative by chromatography in six solvent systems (see Materials and Methods). The two samples migrated identically in each system; the R_f values ranged from 0.2 to 0.9 (Table IV.B). When the 2,4-dinitrophenylhydrazone of the natural material (labelled with ^{32}P) was co-chromatographed in two dimensions (Solvents 3 and 4, see Materials and Methods) with the authentic 2,4-dinitrophenylhydrazone derivative, the coloured material migrated with the radioactivity.

(3) Conversion of the 2,4-dinitrophenylhydrazones to AEP. The natural and synthetic derivatives were converted to AEP by catalytic reduction (Towers, Thompson and Steward, 1954). Aqueous solutions of the two derivatives were shaken vigorously for 16 h in the presence of PtO_2 under 4 atm of hydrogen. Each solution was filtered and applied to a column (1.5 cm x 8 cm) of Dowex I X-8 (acetate) which was washed with water. AEP was identified (Liang and Rosenberg, 1966), in both instances, by its elution from the columns with 0.1 M acetic acid, by its characteristic position on electrophoresis which was identical with a sample of authentic AEP, and by its positive tests on paper with

Table IV.B. Chromatography of the 2,4-dinitrophenylhydrazone derivatives of authentic and isolated 2-phosphonoacetaldehyde

R_f values	Solvent system (see Materials and Methods)					
	1	2	3	4	5	6
Authentic material	0.21	0.23	0.41	0.57	0.83	0.91
Isolated material	0.21	0.23	0.41	0.57	0.83	0.91

ninhydrin and phosphorus reagents. In the case of the natural derivative, the AEP formed was also radioactive.

Infrared and Proton Magnetic Resonance Studies

The infrared spectra of the natural and synthetic compounds in ^2H_2O solution are shown in Fig. IV.6. Apart from a small intensity difference of a band near 1675 cm^{-1}, the spectra are the same, thus confirming the chemical identity of the two samples. There is a prominent carbonyl stretching band, with a frequency for the fully protiated material at about 1708 cm^{-1}. In ^2H_2O solution, the protons on the α-carbon are slowly exchanged, and the carbonyl stretching frequency is then reduced to about 1704 cm^{-1}. There are weak CH stretching bands at about 2925 cm^{-1} and 2865 cm^{-1}. A band near 675 cm^{-1} may be caused by C-P stretching.

The proton magnetic resonance spectra of the natural and synthetic compounds (Fig. IV.7) consist of two multiplets. That at lowest field (τ 0.42) is readily assigned to the aldehyde group, and is split by the adjacent methylene group to form a triplet. The other signal, from the methylene group (τ 6.97), is split by coupling with the aldehyde proton (J = 5 cycles/sec) and with the adjacent phosphorus nucleus (J = 22 cycles/sec) to form a quartet. This provides evidence

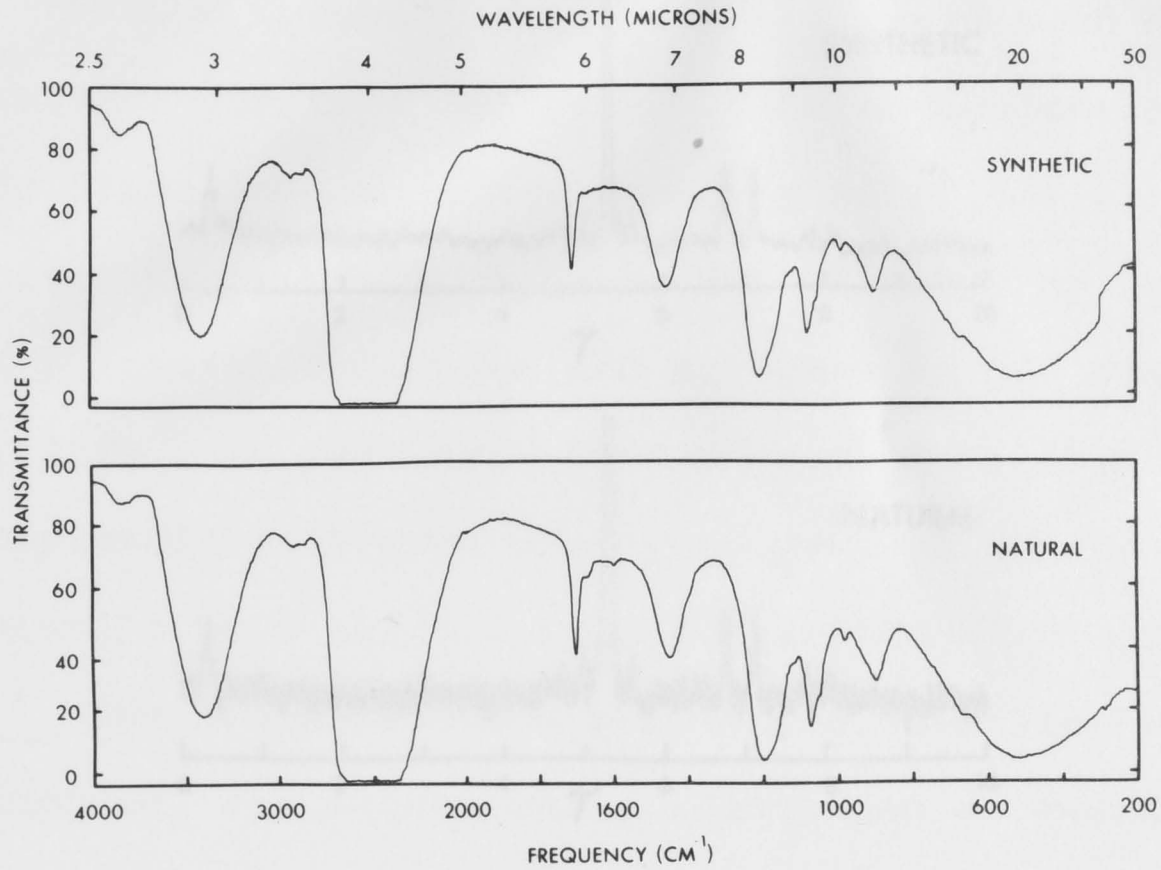


Fig. IV.6 Infrared spectra of synthetic and natural 2-phosphonoacetaldehyde.

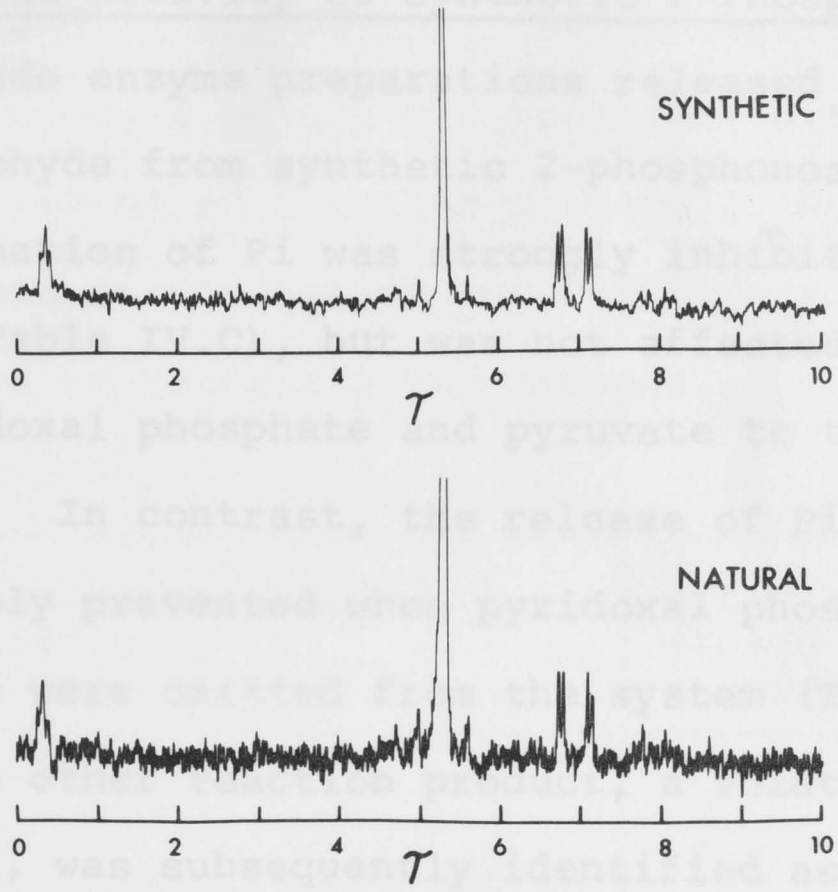


Fig. IV.7. Proton magnetic resonance spectra of synthetic and natural 2-phosphonoacetaldehyde.

for a direct bond between the carbon and phosphorus atoms. On standing overnight, in ^2H_2O, the signal from the methylene group disappeared, indicating that the protons on the α-carbon had been exchanged.

Biological Activity of Synthetic 2-Phosphonoacetaldehyde

Crude enzyme preparations released both Pi and acetaldehyde from synthetic 2-phosphonoacetaldehyde. The formation of Pi was strongly inhibited by orthophosphite (Table IV.C), but was not affected by the addition of pyridoxal phosphate and pyruvate to the reaction mixture. In contrast, the release of Pi from AEP was completely prevented when pyridoxal phosphate and pyruvate were omitted from the system (Table IV.A).

The other reaction product, a volatile carbonyl compound, was subsequently identified as acetaldehyde. It was detected as the 2,4-dinitrophenylhydrazone (see Materials and Methods). The compound was not formed when the enzyme preparation was first boiled, nor when orthophosphite was added to the reaction mixture. Microscopic examination showed that two types of crystal were present (sheaves and needles). The same two types were also obtained, under these conditions, from authentic acetaldehyde. The crystals of the authentic derivative and those of the product from the

Table IV.C. Factors affecting the release of Pi from 2-phosphonoacetaldehyde by a cell-free preparation of B. cereus (AI-2)

The reaction mixture contained 2-amino-2-methyl-1,3-propandiol-HCl buffer, 100 mM (pH 8.4); 2-phosphono-acetaldehyde, 4 mM and the bacterial extract (6 mg protein per ml of reaction mixture). Details of the conditions of incubation and estimation of Pi are described in Materials and Methods.

Additions to reaction mixture	Activity (µmole Pi released per h per mg protein)
None	0.97
Pyridoxal phosphate and sodium pyruvate (both at 0.5 mM)	1.00
Orthophosphite (5 mM)	0.17

reaction mixture were washed with 2 M HCl and then recrystallized from hot ethanol. The presence of two crystal types made the determination of melting points difficult. In both cases, the needles melted at 145-147°, a temperature lower than that generally reported in the literature, but the same as that obtained by De Koning (1966a). The sheaves, when carefully sublimed, melted at 166-168°, the value reported by Vogel (1956). The two derivatives were compared by chromatography. Strips of Whatman No. 3 paper were first dipped in N,N-dimethyl-formamide-ethanol (1:3, by vol.), blotted, and the samples in chloroform solution applied to the moist paper (Spare and Virtanen, 1961). The papers were then placed in jars containing the six solvents mentioned in Materials and Methods. A sample of the 2,4-dinitrophenyl-hydrazone derivative of 2-phosphonoacetaldehyde was included in each run. Although it was not possible to measure R_f values in these systems, the two acetaldehyde derivatives migrated identically in all six solvents, and separated distinctly from the 2-phosphonoacetaldehyde derivative.

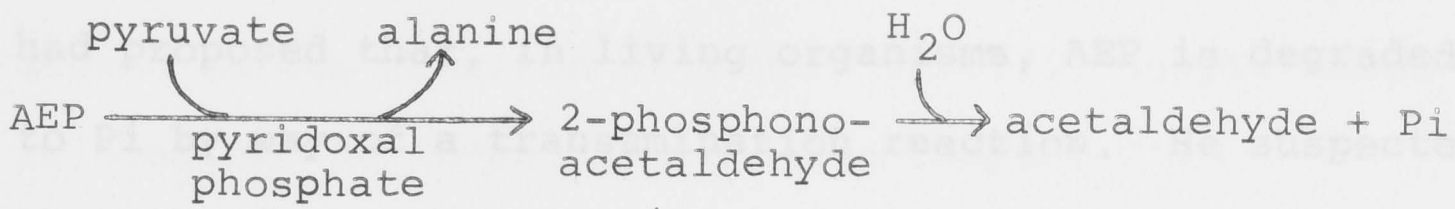
When supplied as a sole source of phosphorus, 2-phosphonoacetaldehyde failed to support the growth of the wild strain (W) of B. cereus, and of the mutant S7-I (see Chapter III), as well as the mutant AI-2.

The Fate of the Carbon Moiety of AEP in Whole Cells

Only preliminary experiments have been carried out with whole cells to determine the fate of the carbon moiety of AEP. When [^{3}H]AEP was metabolized by *B. cereus* (AI-2), most of the ^{3}H-label was rapidly returned to the medium, probably as water (Rosenberg, Liang and La Nauze, 1967). Only a small portion of the ^{3}H-label could be recovered as the dimedone-acetaldehyde complex (Gaffney, Williams and McKennis, 1954). In another experiment, cells of the same mutant, which had been starved of phosphorus for 3 h, were placed in the centre well of a Conway Diffusion Unit and AEP was added to a concentration of 10 mM. It was hoped that, even though *B. cereus* is an aerobic organism, sufficient metabolism of AEP would occur under these conditions to detect whether or not acetaldehyde was released from cells degrading AEP. Thus, 2,4-dinitrophenylhydrazine was added to the outer well, as described in Materials and Methods, and the sealed unit incubated overnight at 30°. A few crystals appeared in the outer well. These were washed in 2 M HCl and chromatographed in solvent system No. 4 (see Materials and Methods) as described above. The material migrated identically with a sample of the 2,4-dinitrophenylhydrazone derivative of authentic acetaldehyde.

DISCUSSION

The work presented in this chapter identifies 2-phosphonoacetaldehyde as an intermediate in the breakdown of AEP to Pi by cell-free extracts from B. cereus (AI-2), thus providing evidence that degradation proceeds by the following pathway :



Further evidence is provided by the degradation of synthetic 2-phosphonoacetaldehyde to acetaldehyde and Pi by the cell-free extract. This reaction is inhibited by orthophosphite and is not dependent on the presence of pyridoxal phosphate and pyruvate. The release of Pi from AEP by the extract is also inhibited by orthophosphite, but, in this case, the reaction does not proceed if pyridoxal phosphate and pyruvate are omitted from the reaction mixture. It also seems likely that this is the pathway whereby AEP is broken down in vivo, in that radioactive bands, migrating in a position similar to 2-phosphonoacetaldehyde, have been detected on electropherograms of extracts from whole cells metabolizing [^{32}P]AEP. Furthermore, the growth

of B. cereus on AEP as a sole source of phosphorus is inhibited if orthophosphite is present (Chapters II and III). As shown above, B. cereus is unable to grow on 2-phosphonoacetaldehyde as a source of phosphorus, presumably because this compound is not transported into the cells.

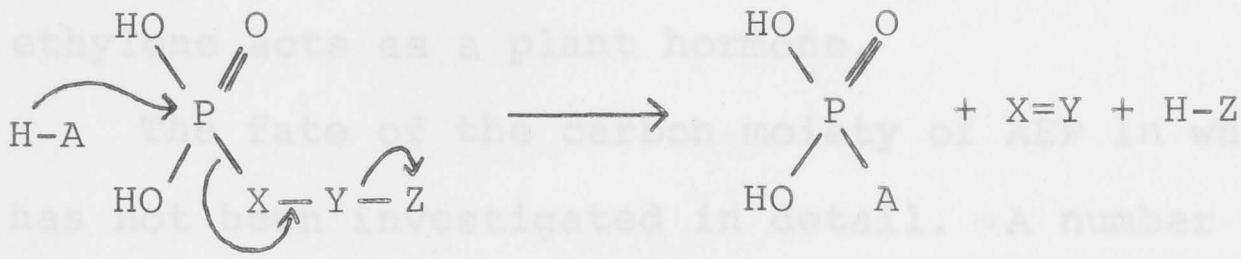
As mentioned in the Introduction, De Koning (1966a) had proposed that, in living organisms, AEP is degraded to Pi by way of a transamination reaction. He suspected that the 2-phosphonoacetaldehyde so formed was sufficiently unstable to decompose spontaneously to Pi and acetaldehyde. The work presented here partly confirms this hypothesis, but also shows that 2-phosphonoacetaldehyde is stable under physiological conditions and that an enzyme is required to catalyse its breakdown.

Recently, Roberts et al. (1968) have shown that extracts from a number of sources catalysed the transamination of a range of aminoalkylphosphonates. Their results indicate that the ability to transaminate these compounds may be fairly widespread, and may represent the action of non-specific transaminases. These workers were unable to demonstrate the release, by preparations from Tetrahymena pyriformis, of ^{32}Pi from [^{32}P]AEP,

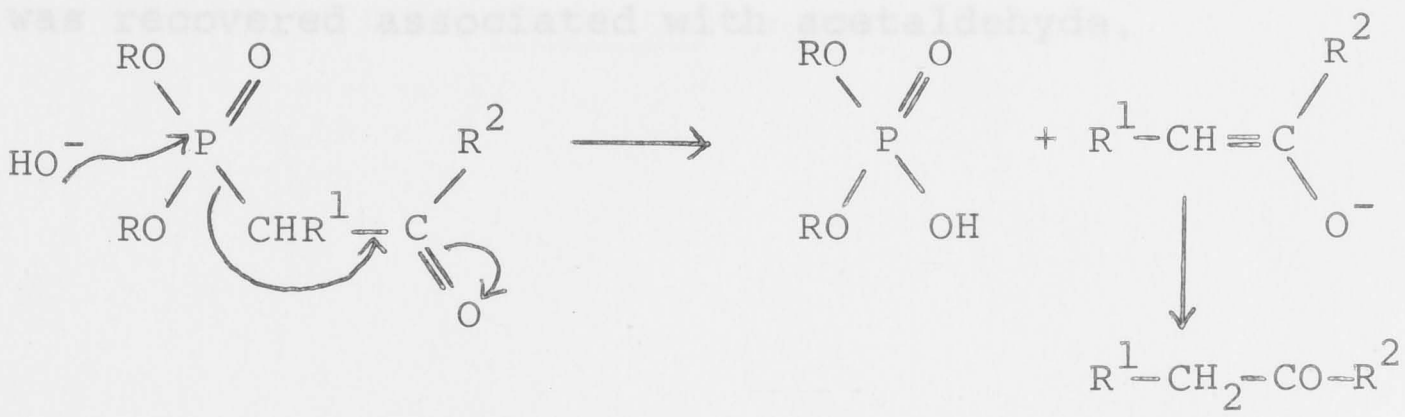
although they were able to isolate an unidentified ^{32}P-labelled 2,4-dinitrophenylhydrazone from the reaction mixture. The failure to detect any liberation of ^{32}Pi from [^{32}P]AEP provides further evidence for the stability of 2-phosphonoacetaldehyde under physiological conditions. The cleavage of the carbon-phosphorus bond of 2-phosphonoacetaldehyde by certain organisms may thus depend on the presence of a more specific enzyme which is confined to these species (see Chapter V).

The formation of 2-phosphonoacetaldehyde, as an intermediate in the degradation of AEP, suggests that the carbon-phosphorus bond is more labile in this compound than in AEP. Isbell, Englert and Rosenberg (1969), who synthesized 2-phosphonoacetaldehyde, found that it hydrolysed at a moderate rate on heating at 90° for 8 h in a buffer at pH 5, although it appeared to be stable in aqueous solutions at 25°, both at low and high pH values. AEP, on the other hand, does not hydrolyse after heating at 120° for 72 h in 6 M HCl (Kandatsu and Horiguchi, 1962). Chavane (1949) stated that the polarity of substituent groups on the alkyl chain can significantly affect the stability of phosphonates and predicted that 2-phosphonoacetaldehyde would be unstable (also see Freedman and Doak, 1957).

Clark, Hutchinson, Kirby and Warren (1964) have pointed out that, in systems designated by them "P-XYZ", the cleavage of the P-X bond requires that the group Z be able to accommodate the electrons of this bond :



For β-ketoalkylphosphonates, Z is a carbonyl group. In alkaline conditions, these compounds undergo hydrolysis to release Pi and the corresponding carbonyl compound (Kreutskamp and Kayser, 1956) according to the following scheme :



Examples of electron-withdrawing groups, other than aldehydes, on the β-carbon of phosphonates resulting in instability of the carbon-phosphorus bond are known. For instance, Maynard and Swan (1963a, b) have reported

that β-halogenoalkylphosphonates are rapidly decomposed, in alkaline solutions, to the corresponding olefin and Pi with the expulsion of the halide ion. Recently, Cooke and Randall (1968) reported that plant tissues hydrolysed 2-chloroethylphosphonate to release ethylene; ethylene acts as a plant hormone.

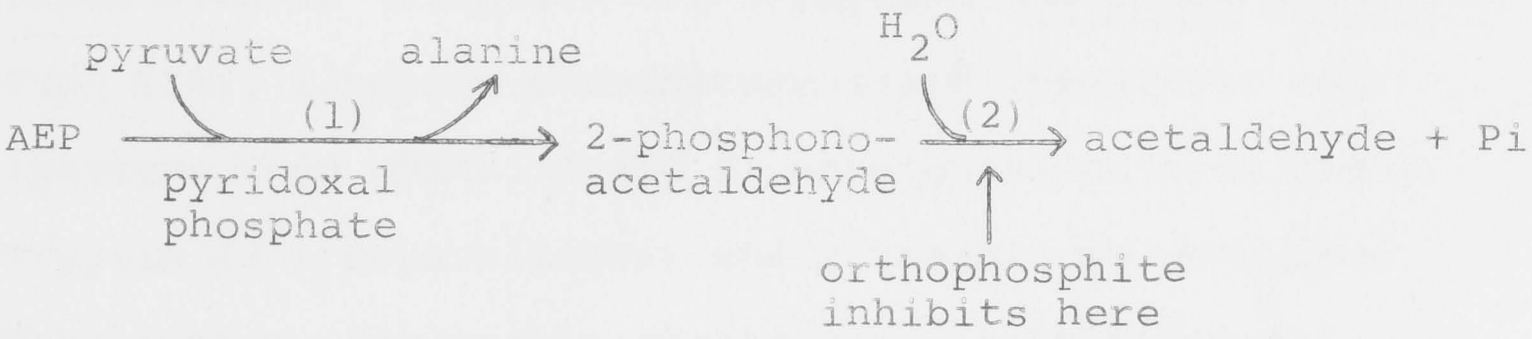
The fate of the carbon moiety of AEP in whole cells has not been investigated in detail. A number of species of bacteria are able to oxidize acetaldehyde to acetate (Jacoby, 1963), which would most probably be further metabolized, via the Kreb's cycle, to CO_2 and H_2O. If this reaction occurs in B. cereus, it would explain why most of the ^3H-label from [^3H]AEP was rapidly lost from the cells and only a small portion was recovered associated with acetaldehyde.

CHAPTER V

The Purification and Properties of "Phosphonatase", an Enzyme which Cleaves the Carbon-Phosphorus Bond, Isolated from *Bacillus cereus*

INTRODUCTION

In the previous chapter, I described the degradation of AEP by cell-free extracts from a strain of Bacillus cereus (AI-2). In crude solutions, the overall reaction showed an obligatory requirement for pyruvate and pyridoxal phosphate, which suggested that AEP was probably transaminated before it was degraded to Pi. The addition of orthophosphite to the cell-free system prevented the release of Pi from AEP and resulted in the accumulation of an intermediate, which was subsequently identified as 2-phosphonoacetaldehyde, thus establishing that, in B. cereus, AEP is degraded in a two-step process :



In this chapter, I describe the purification of the enzyme catalysing the second reaction and some of its properties. The enzyme appears to be similar in a number of ways to the alkaline phosphatase of Escherichia coli (Garen and Levinthal, 1960), but the only phosphate-ester it has been found to cleave is

p-nitrophenylphosphate, and this is at a rate considerably lower than the rate at which it degrades 2-phosphonoacetaldehyde. Conversely, the alkaline phosphatase from E. coli was unable to degrade 2-phosphonoacetaldehyde, even after prolonged incubation with this compound. Enzymes able to cleave the carbon-phosphorus bond have not been reported before, and we have proposed that this enzyme be called 2-phosphonoacetaldehyde phosphonohydrolase[a]. In this thesis, the enzyme will be referred to by the trivial name of "phosphonatase".

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[a]This name has been submitted to the Committee on Enzyme Nomenclature of the International Union of Biochemistry (communicated to E.C. Webb), but no acceptance has yet been received.

MATERIALS AND METHODS

Chemicals

2-Phosphonoacetaldehyde was prepared from 2-acetoxy-2-chloroethylphosphonyldichloride (Isbell, Englert and Rosenberg, 1969); this, AEP and amino-methylphosphonate were provided generously by Professor A.F. Isbell of the Texas A and M University.

Acrylamide, N,N'-methylene-bis-acrylamide and N,N,N',N'-tetramethylenediamine (TEMED) were purchased from Eastman Organic Chemicals. N-Tris(hydroxymethyl)-methyl-2-aminoethane sulphonic acid (TES), p-nitrophenyl-phosphate and yeast alcohol dehydrogenase were obtained from Calbiochem. Ovalbumin (egg white, Grade V), bovine serum albumin (Fraction V), α-amylase (Bacillus subtilis, Type IIA), alkaline phosphatase (calf intestine) and lysozyme (egg white, Grade I) were obtained from Sigma. Trypsin (2 x crystallized) and ribonuclease were from Worthington, deoxyribonuclease was from Nutritional Biochemicals Co. and soybean trypsin inhibitor was from Mann Research Laboratories.

All other chemicals were of analytical grade or of the highest purity available.

Microorganism and Cell-Free Preparations

The wild strain (W) and the mutant strain (AI-2) of Bacillus cereus used in the present work have been described in Chapters II and III, respectively.

Details of the composition of the media used have been given in Chapter II. The procedure adopted for the preparation of cell-free extracts from the mutant strain (AI-2) is described in Results.

Enzyme Assays

Enzyme reactions were carried out at 25°.

For the assay of fractions from columns and for some of the studies on the purified enzyme, the reaction mixture contained : 2-amino-2-methyl-1,3-propandiol-HCl (Ammediol-HCl) buffer, 100 mM (pH 8.5); $MgCl_2$, 5 mM; 2-phosphonoacetaldehyde, 2 mM; and the bacterial extract (about 3-5 µg of the purified enzyme per ml of reaction mixture). After incubation, the reaction was stopped by the addition of 0.75 M perchloric acid, and the Pi released was estimated by the method of Harris and Popat (1954). Any alterations in the concentration of the components of the assay system are indicated in the text.

For kinetic studies, sodium diethylbarbiturate (Veronal)-HCl buffer (pH 8.5) was used instead of

Ammediol-HCl, as the high concentrations of acetaldehyde used in inhibition studies appeared to be interfering with the buffering capacity of Ammediol, presumably by the formation of a Schiff's base complex. In these studies, the Pi released was estimated by the method of Itaya and Ui (1966), which is about ten times more sensitive than that of Harris and Popat, but must be used with some caution (Barrett, Butler and Wilson, 1969). The acetaldehyde released was estimated spectrophotometrically by reducing it to ethanol in the presence of excess NADH and alcohol dehydrogenase (Kaplan and Stadtman, 1968).

One unit of phosphonatase activity is defined as that amount of enzyme which releases 1 μmole of Pi or acetaldehyde per minute from 2-phosphonoacetaldehyde.

Purification of Phosphonatase

The purification of phosphonatase is described in Results. The buffer used throughout this procedure consisted of 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 0.1 mM dithiothreitol, and will be referred to as TMD-buffer.

Protein solutions were concentrated by ultra-filtration using a Diaflo apparatus (Amicon Co.). The pressure was maintained between 40-100 $lb/inch^2$

with helium gas according to the instruction manual. A UM-10 membrane was used; this has an exclusion volume of 10,000 daltons. Unless otherwise stated, protein was measured by the method of Lowry, Rosebrough Farr and Randall (1951), using bovine serum albumin as a standard.

For ammonium sulphate fractionations, the amount of solid ammonium sulphate to be added to the enzyme solution to reach the required saturation was calculated from the nomogram of di Jeso (1968).

A linear gradient (Snyder, 1965) of NaCl in TMD-buffer was applied to elute the protein from DEAE-Sephadex columns. The conductance of fractions was measured with a Phillips conductivity measuring bridge, and the actual gradient obtained was determined by comparison with a standard solution of NaCl prepared in TMD-buffer.

Polyacrylamide Gel Electrophoresis

Gel electrophoresis was carried out essentially as described by Davis (1964). Two buffer systems were used - the Tris-glycine system of Davis (1964; pH range 9-10), and the triethanolamine(TEA)-TES-chloride system of Orr (1969; pH range 7-8). In both cases, gels were polymerised with light using riboflavin

(13.3 μM) as a catalyst. The stacking gel consisted of 2.5% acrylamide and 0.3% bis-acrylamide, and the resolving gel of 9% acrylamide and 0.1% bis-acrylamide. Electrophoresis was carried out at 4°, and the voltage was maintained at 12.5 volts/cm. As indicated in the Results section, $MgCl_2$ (10 mM) was sometimes included in the gels; it was added to the upper buffer reservoir and the gel itself, but not to the lower buffer reservoir.

Molecular Weight Determinations

(1) Gel filtration. The molecular weight of phosphonatase was determined by gel filtration according to the method of Andrews (1964, 1965), using a column of Sephadex G-150 (1.5 cm x 80 cm) equilibrated with TMD-buffer.

(2) Polyacrylamide gels. The molecular weight of phosphonatase was also determined on polyacrylamide gels using a combination of the methods of Zwaan (1967) and Parish (1969). The TEA-TES-chloride buffer system mentioned above was employed, as phosphonatase is unstable at higher pH values. However, this system did have the disadvantage that only proteins with an isoelectric point below pH 6 were suitable as standards. Two gel concentrations (8% and 12%) were prepared from

a stock solution consisting of 20% acrylamide and 0.52% bis-acrylamide; the final gel concentration is expressed with respect to the concentration of monomer only. The gels were cast in glass tubes (0.5 cm x 7.5 cm) instead of a single slab, and no stacking gel was used. The samples were electrophoresed at the same time, and the run terminated when the bromphenol blue marker, added to the upper buffer reservoir, had run to within 1 cm of the end. The gels were removed from the tubes, stained for 3 h with 0.25% (w/v) amidoblack and 0.5% (w/v) mercuric chloride, and destained electrophoretically (Davis, 1964). The ratio of the distance migrated by each protein in the two gel concentrations was determined in three separate experiments and the average of these calculated; the logarithm of this ratio is directly proportional to the molecular weight of the protein (Parish, 1969).

Peptide Mapping

(1) Tryptic digestion. The purified enzyme (8 mg) was denatured by performic acid oxidation (Hirs, 1956). The protein was lyophilised from the performic acid mixture. It was dissolved in 1 ml of 98% (v/v) formic acid, 10 ml of water was added to destroy

residual performic acid and peroxides, and it was then lyophilised again.

The protein was digested with trypsin (treated with L(1-tosylamido-2-phenyl)ethyl chloromethyl ketone to destroy any chymotryptic activity; Schoellmann and Shaw, 1963) at 37° in 0.5% (w/v) NH_4HCO_3, pH 8.0, for 4 h. The digest was lyophilised to remove the NH_4HCO_3, and the residue re-dissolved in 4 ml of water. One drop of glacial acetic acid was added to bring the pH to approximately 4, and the solution allowed to stand for 15 min. The heavy precipitate, or insoluble "core", which formed was removed by centrifugation and was washed several times with water containing one drop of glacial acetic acid (approximate pH of 4). The soluble material (plus washings) was lyophilised and stored in this form at -20°; the core was stored as the centrifuged precipitate.

(2) Electrophoresis and chromatography. Electrophoresis was carried out in a high voltage electrophoresis apparatus similar to that of Ryle, Sanger, Smith and Kitai (1955), with mineral turpentine as the inert coolant. Samples of the soluble peptides (about 2 mg) were applied to Whatman No. 3 MM paper and run in formic-acetic acid buffer, pH 1.9 (87 ml glacial

acetic acid plus 25 ml formic acid (88%) to one l; Atfield and Morris, 1960), or in pyridine-acetic acid buffer, pH 4.7 (25 ml pyridine plus 25 ml glacial acetic acid to one l; Schwartz, 1963). A sample of the core peptides was run in 2% (w/v) $(NH_4)_2CO_3$, pH 8.9, with a sample of the soluble peptides run in parallel to see whether there was any contamination of the fraction by these peptides. After the electropherograms had been dried at room temperature, the strips of paper containing the separated peptides were sewn onto fresh sheets of paper (Milstein and Sanger, 1961) and then chromatographed in the second dimension using either n-butanol:acetic acid:water (4:1:5, by vol., upper phase only; Katz, Dreyer and Anfinsen, 1959), or iso-amyl alcohol:pyridine:water (35:35:30, by vol.; Wittmann and Braunitzer, 1959).

(3) Specific stains. Each chromatogram was sprayed with ninhydrin reagent (0.1% (w/v) ninhydrin in acetone containing 0.2% (v/v) glacial acetic acid and 0.2% (v/v) pyridine), and stored in the dark for 24 h to allow the colour to develop fully. The chromatograms were photographed and the ninhydrin-positive spots marked lightly with pencil, before another stain was used.

Larger peptides, which stain poorly with ninhydrin, were detected on the chromatograms by chlorination followed by starch-iodide reagent (Rydon and Smith, 1952). Peptides containing tyrosine were detected as described by Easley (1965). Histidine-containing peptides were detected using the Pauly reagent, modified by Milstein (personal communication to D.C. Shaw) as follows : The ninhydrin colour was bleached by dipping the chromatograms through 1% (v/v) HCl in acetone. The following solutions were prepared and cooled separately in ice : A. 1 g sulphanilic acid plus 100 ml 1 M HCl; B. 5% (w/v) sodium nitrite; C. 10% (w/v) sodium carbonate. One volume of A was mixed with one volume of B, and allowed to stand for 10 min in ice, then this was mixed with two volumes of C and immediately sprayed lightly over the chromatogram. The histidine-containing peptides stained red-orange; tyrosine-containing peptides stained brown with this reagent, but they are harder to distinguish than those containing histidine.

Amino Acid Analyses

The protein samples were hydrolysed in sealed, evacuated tubes in 6 M HCl at 110° for 22 h (Crestfield, Moore and Stein, 1963). After removal of the HCl by rotary evaporation under reduced pressure, the hydroly-

sate was chromatographed on a Beckman amino acid analyser, model 120B. The long column was 50 cm high and the short column was 12 cm; the buffer flow rate was 40 ml per h.

The content of tryptophan in the samples was not estimated.

The amino acid composition of individual peptides was determined by cutting out the particular peptide from a chromatogram which had been sprayed only lightly with ninhydrin (0.01% (w/v) in acetone). The peptide was eluted from the paper as quantitatively as possible, the resulting solution was filtered and hydrolysed for analysis as described above.

RESULTS

Purification of Phosphonatase

Early attempts to purify phosphonatase were hampered by a rapid loss of activity, especially during fractionation on Sephadex columns. Subsequently, it was found that Mg^{++} protected against losses of activity and that EDTA in the buffer system (originally added to crude extracts to protect the transaminase carrying out the conversion of AEP to 2-phosphonoacetaldehyde) was actually deleterious. Eventually, the buffer used throughout the purification procedure contained 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$ and 0.1 mM dithiothreitol (referred to as TMD-buffer), and this resulted in good recoveries from columns and enabled enzyme preparations to be stored at -20° for several months with only a gradual fall in activity; however, solutions which were repeatedly frozen and thawed rapidly lost activity.

In the last chapter, it had been assumed that the specific activity of the breakdown enzyme(s) in the mutant AI-2, like that of the transport system, increased after phosphorus-starvation. Following the identification of 2-phosphonoacetaldehyde as the intermediate in the degradation of AEP by this organism,

and the demonstration that an enzyme was required to catalyse its breakdown under physiological conditions, it was now possible to test this hypothesis. Cells were grown overnight at 30° in PPYG medium (see Chapter II). They were then shaken vigorously for various periods of time in the phosphorus-free medium (BXPG-NEM; see Chapter II) modified slightly as follows to contain : 0.25% (w/v) of the amino acid-salt mixture (this was found to be as effective as the 1% concentration previously used) plus 0.5% (w/v) glucose in 50 mM N-ethylmorpholine-HCl buffer (pH 7.5). The cells were then centrifuged and resuspended in 4 vol. of cold TMD-buffer. They were broken in a Sorvall Ribi Fractionator at 25,000 lb/inch^2, and the homogenate centrifuged at 4° for 15 min at 38,000 x g. This experiment (Fig. V.1) confirmed that the specific activity of phosphonatase does, indeed, increase after phosphorus-deprivation, and a 3 h period of starvation was routinely adopted for all further preparations. The use of the mutant (AI-2) instead of the wild strain (W) for the preparation of cell-free extracts has the obvious advantage that induction of phosphonatase is not necessary. In addition, the best cell-free preparations from the mutant have had more than ten

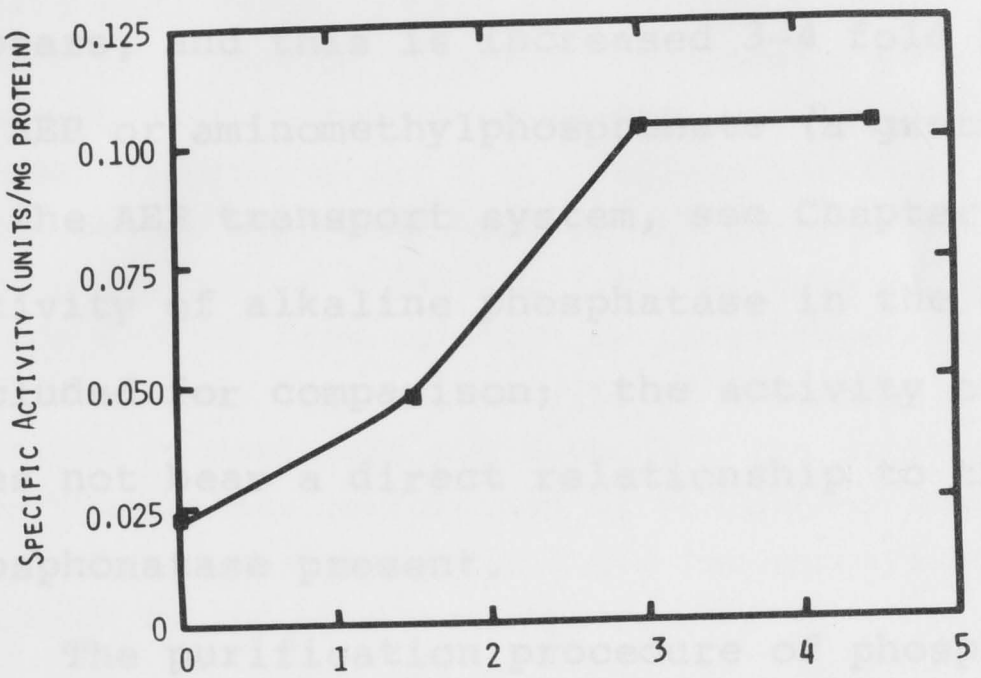


Fig. V.1. The effect of phosphorus-starvation on the activity of phosphonatase in crude, cell-free extracts prepared from the mutant strain (AI-2) of B. cereus. Cells were grown in PPYG medium and resuspended in the modified, phosphorus-free medium (see text) and shaken vigorously. At various times, the cells were removed by centrifugation, cell-free extracts prepared, and the activity of phosphonatase measured (see Materials and Methods). Protein was measured by the Biuret method.

times the activity of those prepared from the wild strain. Table V.A shows the activity of phosphonatase found in extracts prepared from the wild strain after the cells had been treated in various ways. No phosphonatase activity is present in PPYG-grown cells; after prolonged phosphorus-deprivation, some activity appears, and this is increased 3-4 fold by the presence of AEP or aminomethylphosphonate (a gratuitous inducer of the AEP transport system, see Chapter II). The activity of alkaline phosphatase in the cells is included for comparison; the activity of this enzyme does not bear a direct relationship to the amount of phosphonatase present.

The purification procedure of phosphonatase from the mutant AI-2 is shown in Table V.B. All operations were carried out at 4°. Following the removal of cell debris from the crude cell extract by centrifugation, most of the nucleic acids were removed by adding 0.2 volumes of a 0.5% (w/v) solution of protamine sulphate (Step No. 2). A dilute solution of protamine sulphate was used, as phosphonatase began to co-precipitate at higher concentrations. Any nucleic acids remaining in solution were digested by adding ribonuclease and deoxyribonuclease, each to a final concentration of

Table V.A. Phosphonatase activity in crude, cell-free extracts prepared from the wild strain (W) of *B. cereus*.

Cells were grown in PPYG medium, and a cell-free extract prepared from a portion of these. The rest were resuspended in a phosphorus-free medium (BXPG-NEM, see Chapter II) and were shaken vigorously for 2 h. Various additions, as indicated below, were then made to the suspensions, and the cells were shaken for a further 2 h. At this stage, the cells were removed from solution by centrifugation, cell-free extracts prepared, and the activity of phosphonatase measured as described in Materials and Methods. The activity of alkaline phosphatase (determined by estimating the release of Pi from β-glycerophosphate) is shown for comparison. Protein was measured by the Biuret method.

Treatment	Specific Activity (μmole Pi released/min/mg protein)	
	Phosphonatase	Alkaline phosphatase
PPYG-grown cells	0	0.008
2 h-starved cells, treated for a further 2 h as follows :		
no additions	0.0018	0.0132
0.5 mM AEP	0.0061	0.0032
0.5 mM aminomethylphosphonate	0.0048	0.0061

Table V.B. Purification of phosphonatase from a crude cell extract of *B. cereus* (AI-2)

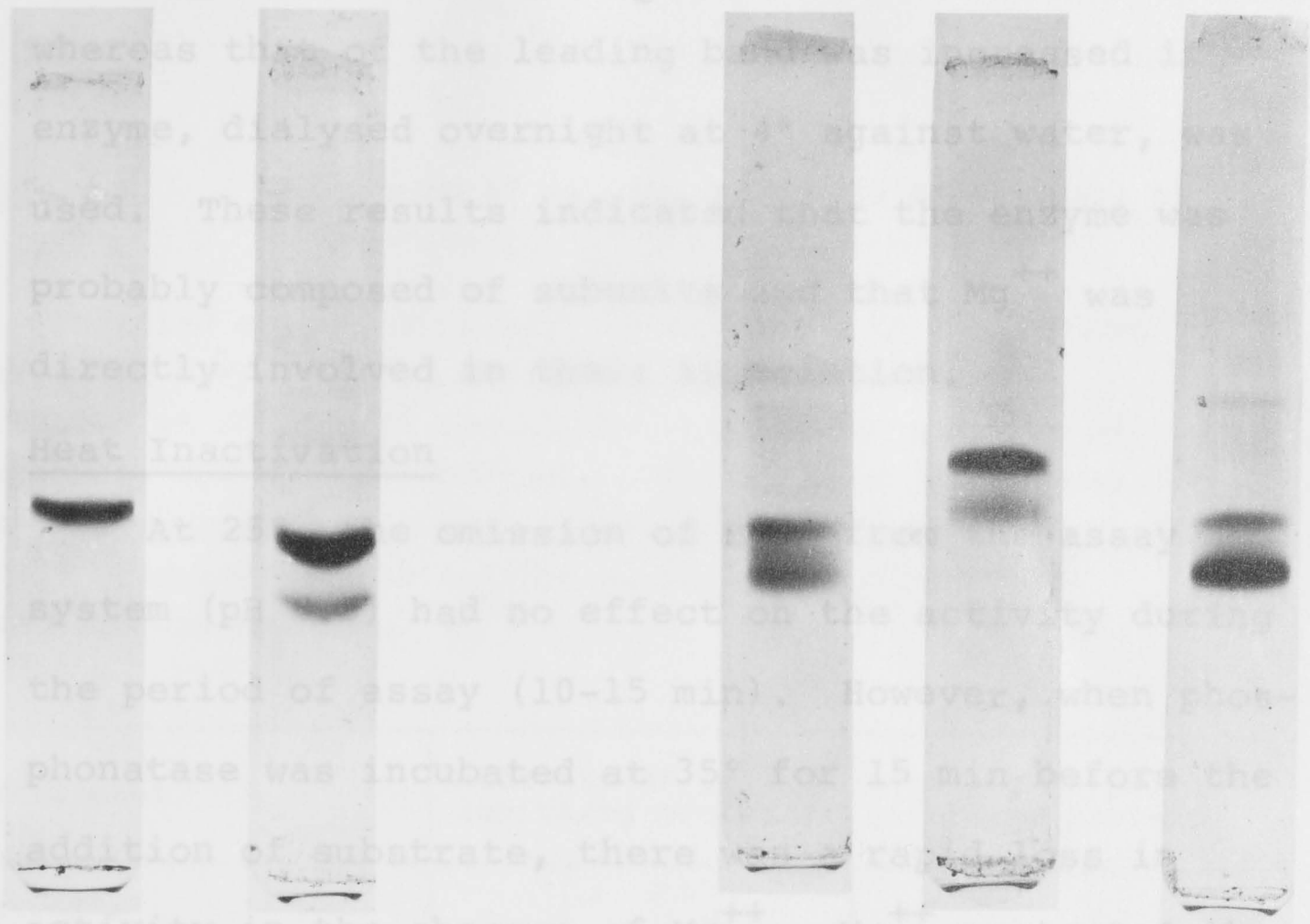
Purification Step	Volume (ml)	Protein (mg)	Activity (units/ml)	Total Activity (units)	Specific Activity	% Recovery	Purification
1. Crude extract	425	6820	0.43	184	0.03	100	1.0
2. Removal of nucleic acids	465	5130	0.34	159	0.03	85	1.0
3. $(NH_4)_2SO_4$ precipitation	48	1290	0.29	140	0.11	76	3.7
4a. DEAE-Sephadex column 1	182	47.3	0.74	135	2.86	74	95
4b. DEAE-Sephadex column 2	114	30.8	0.94	107	3.46	58	115
5. Sephadex G-150 column	53	8.0	1.32	70	8.8	38	292

1 µg/ml, and allowing the solution to stand overnight at 4°. The resulting nucleotides were removed in the subsequent ammonium sulphate precipitation and dialysis which followed this step. Pilot studies with ammonium sulphate showed that phosphonatase did not precipitate sharply, but came out of solution between 0.4 and 0.7 saturation. However, this step (No. 3) was included in the purification procedure as it resulted in the removal of a large amount of contaminating material and a convenient reduction in volume. After dialysis against TMD-buffer containing 0.25 M NaCl, the solution was applied to a column of DEAE-Sephadex equilibrated with the same buffer. The protein was eluted using a linear gradient of NaCl (0.25-0.45 M). The effluent was collected in 13.5-ml fractions which were assayed for enzyme activity and protein content. The fractions comprising the peak of activity were pooled, concentrated and dialysed against 0.3 M NaCl (Step No. 4a). Phosphonatase absorbed strongly to DEAE-Sephadex, and was eluted with 0.38 M NaCl well towards the end of the protein profile. This step resulted in the largest single increase in specific activity during the purification procedure. Since the recovery of enzymic activity was also good, this step was repeated using a

similar column, but a gradient of 0.3-0.4 M NaCl (Step No. 4b). The fractions containing phosphonatase activity were concentrated to a volume of 5 ml and applied to a column of Sephadex G-150 (3 cm x 96 cm) equilibrated in TMD-buffer (Step No. 5), the effluent was collected in 2.5 ml fractions, and those comprising the peak of activity were pooled and stored at -20°. The enzyme was purified as rapidly as possible, as it was shown to be in slow equilibrium with a dissociated form which is removed by each step in the procedure.

Behaviour on Polyacrylamide Gels

Freshly isolated enzyme (from Step No. 5, Table V.B) ran as a single band in polyacrylamide gels using the TEA-TES-chloride buffer system (pH 7-8; Fig. V.2); a minor contaminating band(s) was sometimes seen running behind this band. If the enzyme solution was kept at 4° for about 24 h, subsequent electrophoresis showed that a second, faster-moving component had appeared. Since only the slower band possessed enzymic activity, this suggested that some dissociation had taken place during this time. When the enzyme was electrophoresed in polyacrylamide gels at the higher pH (pH 9-10), diffuse protein appeared between the two bands. The relative intensity of the trailing band was



A B C D E

Fig. V.2. Polyacrylamide gel electrophoresis of phosphonatase. Purified phosphonatase (from Step 6, Table V.B) was electrophoresed on polyacrylamide gels, using either the TEA-TES-chloride system (Tubes A and B) or the Tris-glycine system (Tubes C, D and E) as described in Materials and Methods.

A : Freshly purified enzyme; B : 24 h-old enzyme;
C : 24 h-old enzyme; D : 24 h-old enzyme with 10 mM $MgCl_2$ included in the gel and upper reservoir buffer;
E : enzyme dialysed overnight against water.

increased when 10 mM $MgCl_2$ was included in the system, whereas that of the leading band was increased if enzyme, dialysed overnight at 4° against water, was used. These results indicated that the enzyme was probably composed of subunits and that Mg^{++} was directly involved in their association.

Heat Inactivation

At 25°, the omission of Mg^{++} from the assay system (pH 8.5) had no effect on the activity during the period of assay (10-15 min). However, when phosphonatase was incubated at 35° for 15 min before the addition of substrate, there was a rapid loss in activity in the absence of Mg^{++}. Mg^{++} protected the enzyme against this loss of activity, and the degree of protection was related to the concentration of Mg^{++} present (Table V.C). Inactivation in the absence of Mg^{++} was aggravated when either EDTA or Ca^{++} was also added. To test whether Mn^{++} and Zn^{++} could substitute for Mg^{++}, it was necessary to lower the pH, as at pH 8.5 these ions form insoluble hydroxides. At pH 6.5, the rate of inactivation in the absence of Mg^{++} was much lower than at the higher pH; Mn^{++} did show a slight protective effect, but Zn^{++} and Ca^{++} were antagonistic. As mentioned above, the dissociation of

Table V.C. The effect of various compounds on the rate of inactivation of phosphonatase at 35°

Purified enzyme (5 μg/ml), in either 50 mM Ammediol-HCl buffer (pH 8.5) or in 50 mM K-TES buffer (pH 6.5), was incubated at 35° for 15 min in the presence of a number of compounds, but without substrate. The samples were cooled, 1 mM 2-phosphonoacetaldehyde added, and the activity assayed at 25° by measuring the release of Pi as described in Materials and Methods.

Treatment	Activity at pH 8.5 (units/mg protein)	Activity at pH 6.5 (units/mg protein)
Control :		
Enzyme (not heated) with 5 mM $MgCl_2$	3.40	0.91
Enzyme heated without $MgCl_2$	0.44	0.47
Enzyme heated with :		
0.75 mM $MgCl_2$	1.39	-
2 mM $MgCl_2$	2.00	-
5 mM $MgCl_2$	2.54	0.47
10 mM $MgCl_2$	2.83	-
Enzyme heated with :		
2 mM EDTA	0	-
5 mM $CaCl_2$	0.21	0.04
5 mM $MnCl_2$	-	0.60
5 mM $ZnCl_2$	-	0.06

phosphonatase was greater in polyacrylamide gels run in the buffer system with the pH range of 9-10, than that of pH 7-8. A gradual restoration of activity of the heat-treated enzyme occurred following incubation with 5 mM Mg^{++}. The concentration of the inactive component remaining (probably the dissociated form of the enzyme) decreased exponentially with time (Fig. V.3); 91% of the activity of a sample heated in the presence of 5 mM Mg^{++} was recovered after 90 min.

Molecular Weight Determinations, Peptide Mapping and Amino Acid Analyses

The molecular weight of phosphonatase was estimated to be about 83,000 daltons from studies using Sephadex G-150 (Fig. V.4), and about 70,000 daltons using acrylamide gels (Fig. V.5). The differences in the values obtained by these two methods (which actually measure the ionic radius, not molecular weight) may reflect the effect on the enzyme of alterations in the pH and the ionic environment (cf. alkaline phosphatase; Applebury and Coleman, 1969).

The purified enzyme was digested with trypsin and prepared for peptide mapping as described in Materials and Methods. The soluble peptides were chromatographed in three alternative, two-dimensional systems (see

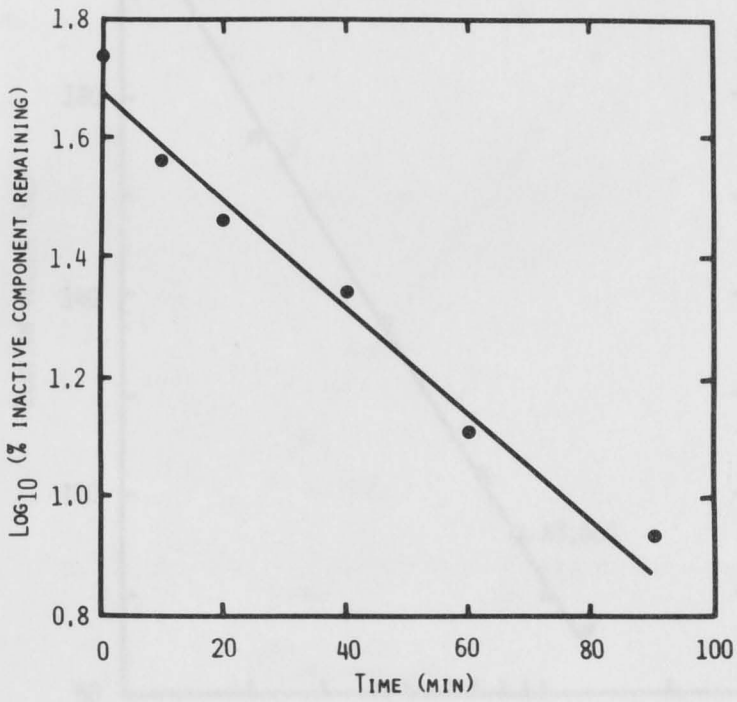


Fig. V.3. Recovery of activity of heat-treated phosphonatase after incubation with $MgCl_2$. Purified enzyme (5 μg/ml) in 50 mM Ammediol-HCl buffer (pH 8.5) was incubated at 35° for 15 min. 5 mM $MgCl_2$ was then added, and the samples incubated at 25° for varying amounts of time before the addition of substrate (zero time). The amount of Pi released after 5 min was estimated as described in Materials and Methods, and the results plotted (by reference to a control heated in the presence of 5 mM $MgCl_2$) as the $\log_{10}$ (% inactive component remaining) v. time of preincubation with $MgCl_2$.

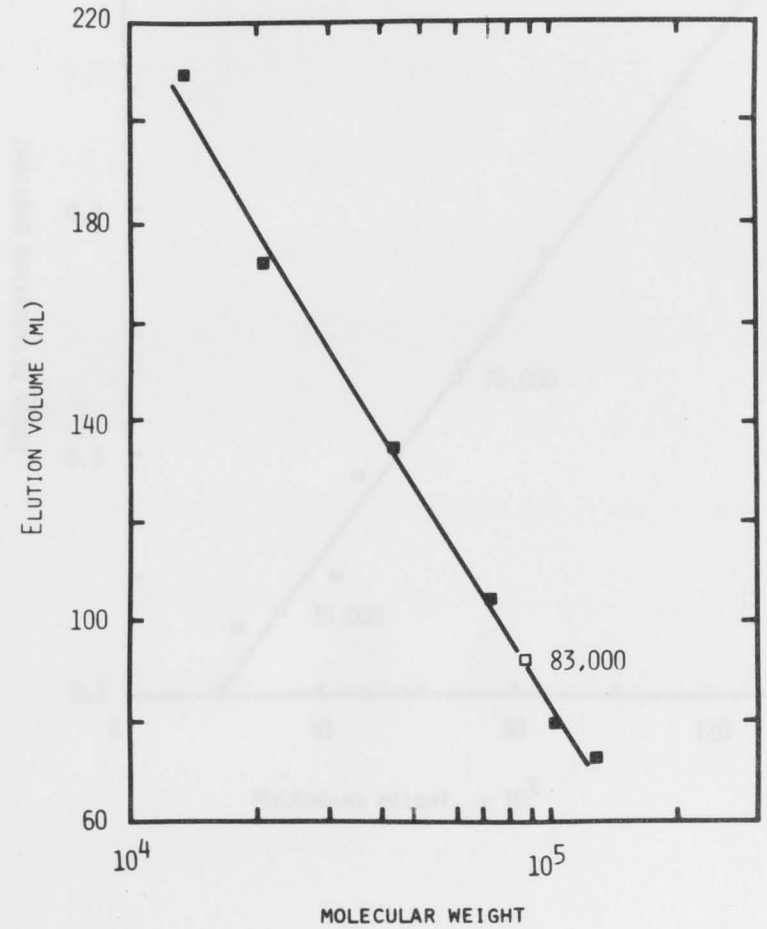


Fig. V.4. Determination of the molecular weight of phosphonatase using Sephadex G-150. Details of the method are described in Materials and Methods. The proteins (■) used as standards were as follows (the molecular weight in daltons and the source of reference are given in brackets); lysozyme (14,000; Altman and Dittmer, 1964); trypsin (21,000; Altman and Dittmer, 1964); ovalbumin (44,000; Altman and Dittmer, 1964); bovine serum albumin (70,000; Klainer and Kelgeles, 1956); alkaline phosphatase from calf intestine (100,000; Engstrom, 1961); yeast alcohol dehydrogenase (129,000; Armstrong, Coates and Morton, 1963). The molecular weight of phosphonatase (□) was estimated to be about 83,000 daltons.

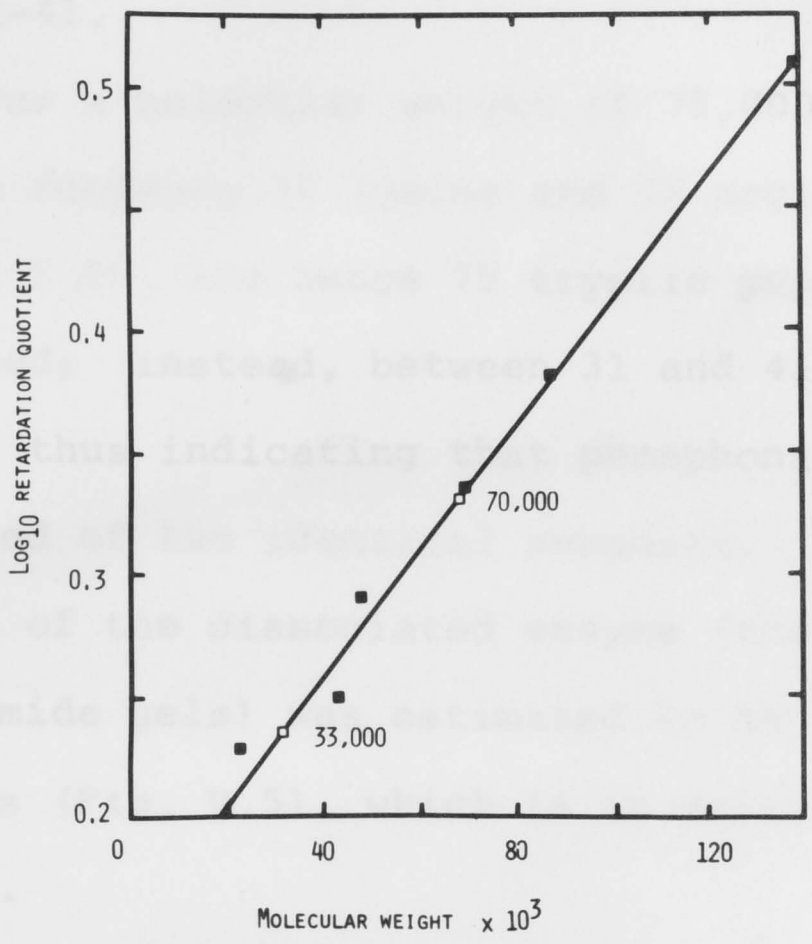


Fig. V.5. Determination of the molecular weight of phosphonatase using polyacrylamide gels. Details of the method are described in Materials and Methods. The proteins (■) used as standards were as follows (the molecular weight in daltons and the source of reference are given in brackets) : soybean trypsin inhibitor (21,500; Wu and Scheraga, 1963); ovalbumin monomer (44,000; Altman and Dittmer, 1964); α-amylase (48,000; Connellan, 1968); bovine serum albumin (70,000; Klainer and Kelgeles, 1956); ovalbumin dimer (88,000) and bovine serum albumin dimer (140,000). The molecular weight of phosphonatase (□) was estimated to be about 70,000 daltons for the active enzyme, and 33,000 for the subunit.

Figs. V.6, 7 and 8) and the core fraction treated separately (Fig. V.9). From a comparison of these, the total number of peptides (including those of the core) was 31-41.

For a molecular weight of 75,000 daltons, the enzyme contains 37 lysine and 37 arginine residues (Table V.D), and hence 75 tryptic peptides would be expected; instead, between 31 and 41 peptides were found, thus indicating that phosphonatase is probably composed of two identical subunits. The molecular weight of the dissociated enzyme (the leading band on acrylamide gels) was estimated to be about 33,000 daltons (Fig. V.5), which is in agreement with this result.

Each of the chromatograms of the soluble peptides was then sprayed with a specific stain. On the first chromatogram, one of the faint ninhydrin-staining peptides stained heavily with the chlorine-starch-iodide reagent. On the second map, five to six peptides containing tyrosine were found, and on the third map, four to five containing histidine. One of the peptides of the core fraction stained heavily with the chlorine-starch-iodide reagent, and this peptide will be referred to as the main core peptide. There was not

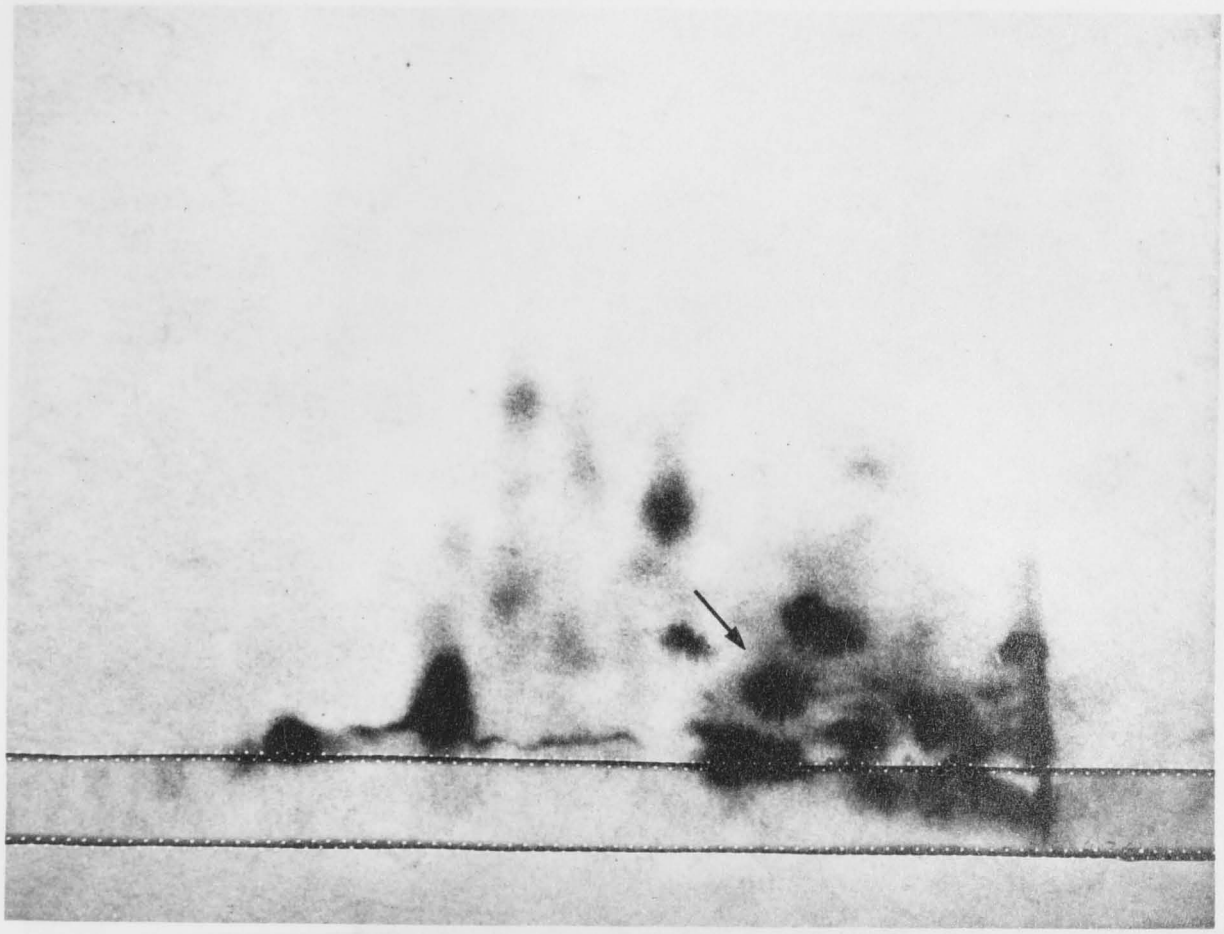


Fig. V.6. Peptide map of the soluble fraction of a tryptic digest of phosphonatase (see text). The chromatogram was electrophoresed in the first dimension at pH 4.7 at 40 volts/cm for 1 h, and then chromatographed in the second dimension in pyridine:iso-amyl alcohol:water (35:35:30, by vol). The chromatogram was stained with ninhydrin as described in Materials and Methods. One of the peptides staining faintly with ninhydrin stained heavily with chlorine-starch-iodide spray, and is indicated by an arrow. This map was kindly prepared by Dr. D.C. Shaw.

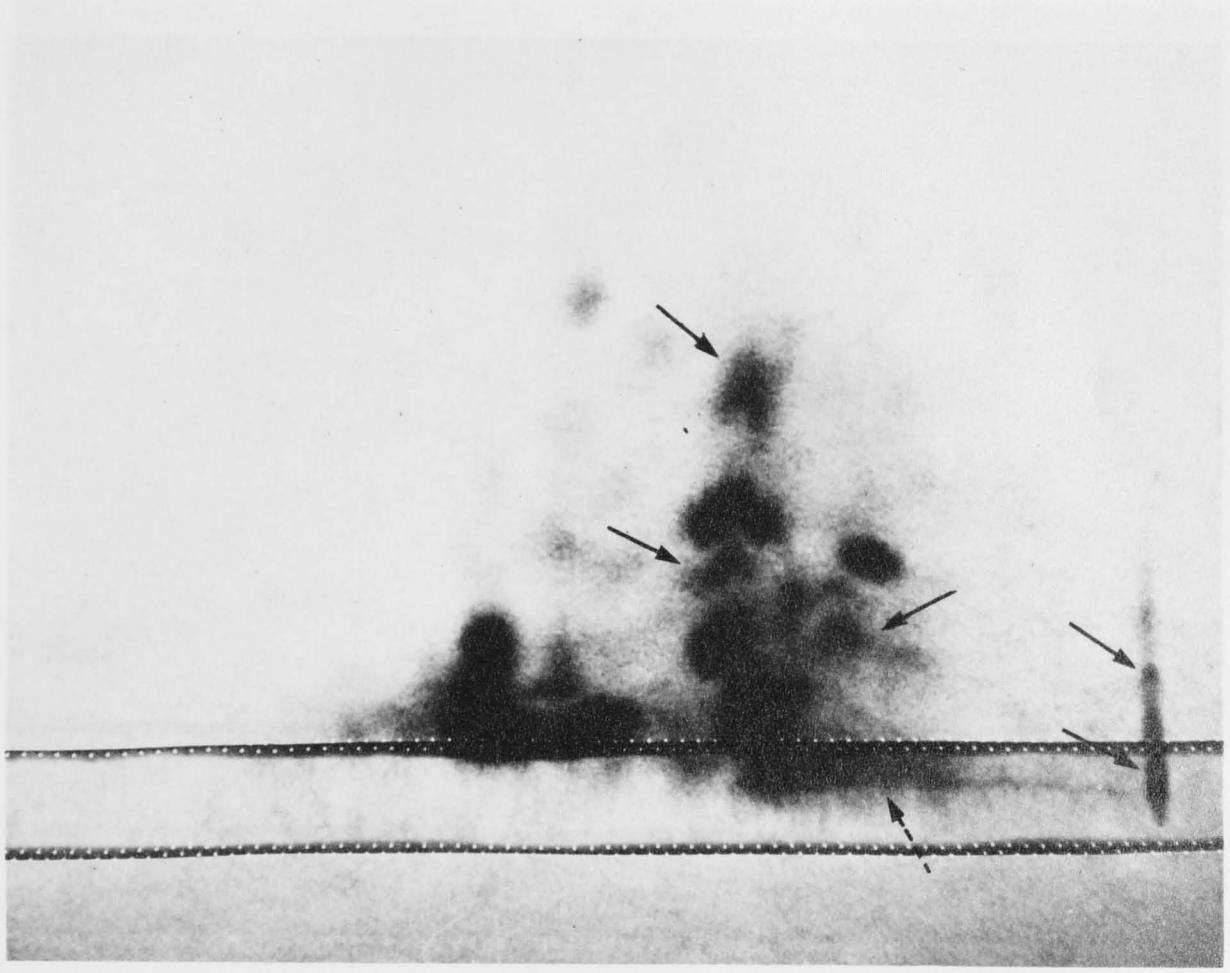


Fig. V.7. Alternative peptide map of the soluble fraction of a tryptic digest of phosphonatase (see text). The chromatogram was electrophoresed in the first dimension at pH 1.9 at 40 volts/cm for 45 min, and then chromatographed in the second dimension in pyridine:iso-amyl alcohol:water (35:35:30, by vol). The chromatogram was first sprayed with ninhydrin (shown here), and then with a specific stain for tyrosine (see Materials and Methods). Those peptides containing tyrosine are indicated by arrows.

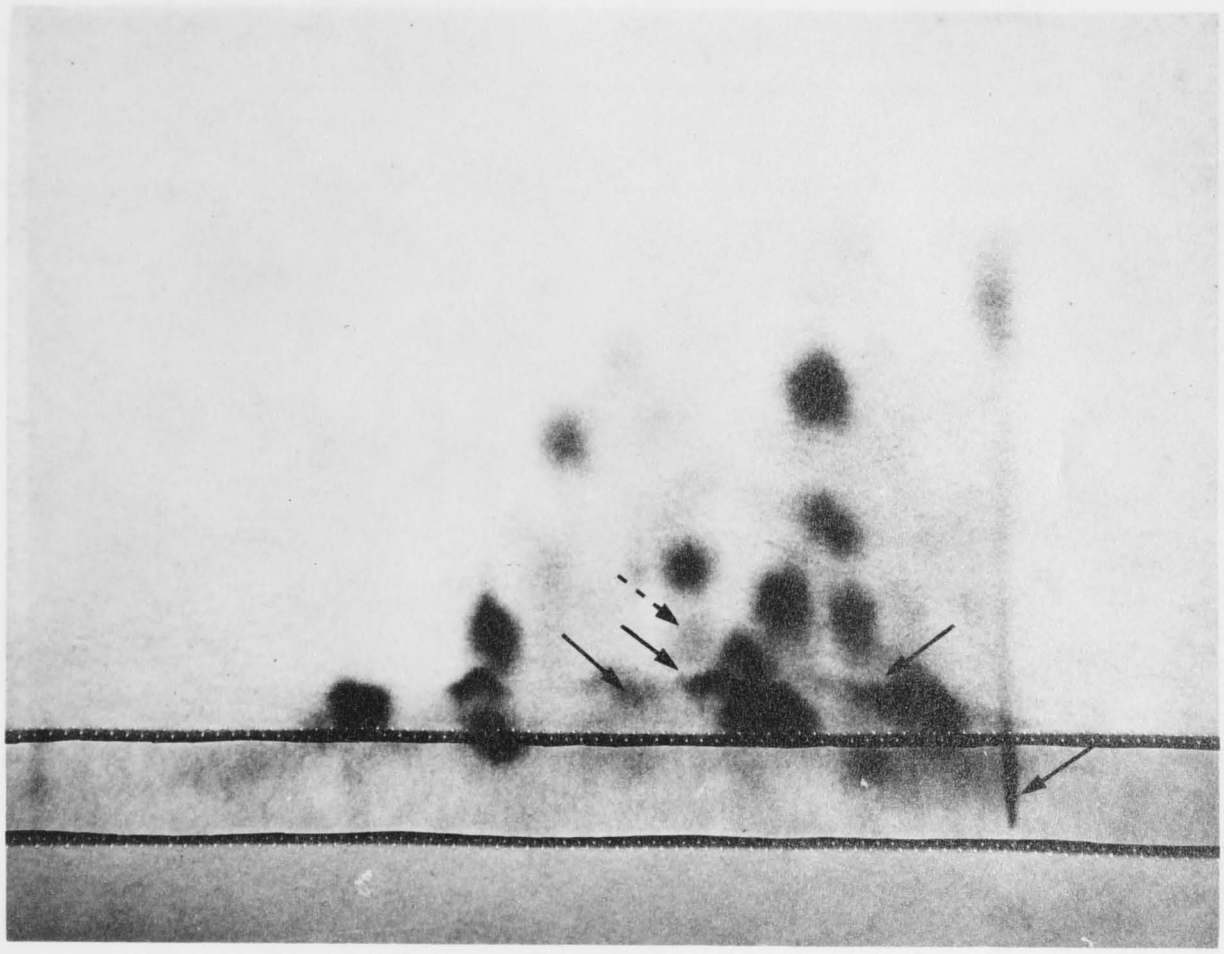


Fig. V.8. Alternative peptide map of the soluble fraction of a tryptic digest of phosphonatase (see text). The chromatogram was electrophoresed in the first dimension at pH 4.7 at 40 volts/cm for 1 h, and then chromatographed in the second dimension in n-butanol:acetic acid:water (4:1:5, by vol, using the upper phase). The chromatogram was first sprayed with ninhydrin (shown here) and then with a specific stain for histidine (see Materials and Methods). Those peptides containing histidine are indicated by arrows.

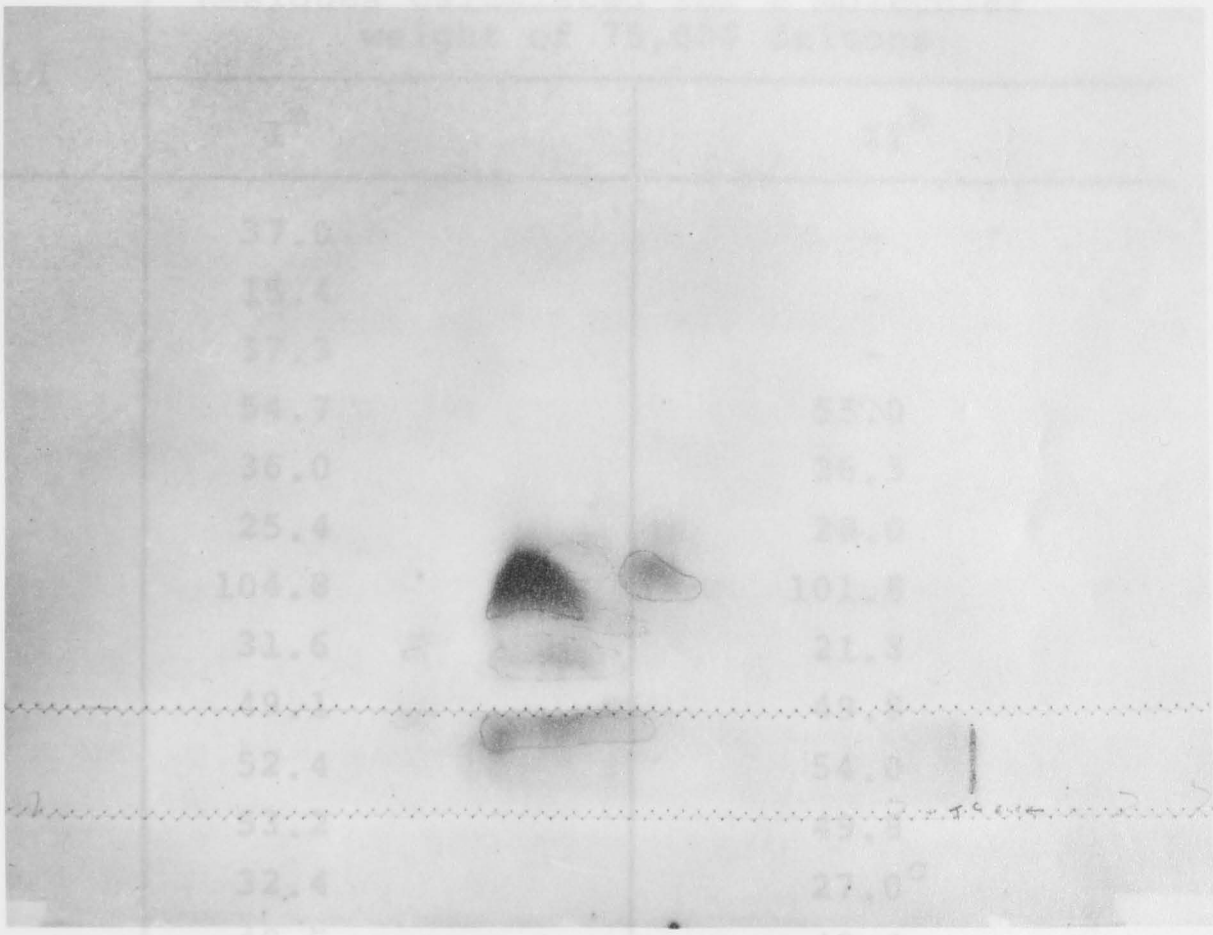


Fig. V.9. Peptide map of the "core" fraction of a tryptic digest of phosphonatase (see text). The chromatogram was electrophoresed in the first dimension at pH 8.9 at 40 volts/cm for 45 min, and then chromatographed in the second dimension in pyridine:iso-amyl alcohol:water (35:35:30, by vol). The peptides staining with ninhydrin were marked lightly with a pencil before the chromatogram was sprayed with chlorine-starch-iodide reagent (shown here) as described in Materials and Methods. This map was kindly prepared by Dr. D.C. Shaw.

Table V.D. The amino acid composition of phosphonatase

Amino acid	Residues calculated for a molecular weight of 75,000 daltons	
	I[a]	II[b]
Lys	37.0	-
His	15.4	-
Arg	37.3	-
Asp	54.7	55.0
Thr	36.0	36.3
Ser	25.4	28.0
Glu	104.8	101.8
Pro	31.6	21.8
Gly	49.1	49.8
Ala	52.4	54.0
Val	53.2	49.8
Met	32.4	27.0[c]
Ileu	48.5	43.6
Leu	47.8	47.8
Tyr	17.5	18.7[d]
Phe	29.9	27.0
½ Cys	-	-
Cysteic		5.5[e]

[a]Single 22 h hydrolysate; 2 mg purified enzyme.

[b]Duplicate 22 h hydrolysates of 75 µg of performic acid-oxidized material, estimated using the expanded scale. This was a different preparation from I.

[c]As the methionine sulphone.

[d]Determined from an unoxidized sample of II run in parallel.

[e]The unoxidized material had a peak corresponding to 3.5 residues of cysteic acid/mole. This may represent oxidized material, as the sample was several months old, or it may be another compound eluting with cysteic acid.

enough material to do additional peptide maps of the core fraction for the specific stains.

The amino acid analyses of the core fraction and the main core peptide are given in Table V.E. Interestingly, the main peptide contains no basic amino acids, and it is thus probably the C-terminal peptide. It also contains a large amount of glutamic acid, which would explain why the peptide had limited solubility at pH 4, but migrated well on electrophoresis at pH 8.9 (Fig. V.9). The core and the main peptide each contained about 2.8% tyrosine. Assuming that there is one tyrosine in the main core peptide, and that this peptide constitutes 50% of the core, this will give a minimum of two tyrosine residues in the core fraction. Histidine was also present at 2.8% in the whole fraction, though not in the main peptide, and hence, on the above reasoning, there is a minimum of two histidine residues in the core. Thus, altogether, between 7 and 8 tyrosine-containing peptides, and between 6 and 7 histidine-containing peptides were found. The amino acid analysis of the whole protein shows that there are 18 tyrosine and 15 histidine residues per molecular weight of 75,000 daltons. Assuming that these residues occur more often than not in separate tryptic-peptides, then a little

Table V.E. Amino acid composition of the "core" and the main "core" peptide of a tryptic digest of phosphonatase (see text)

Amino acid	Moles %[a]	
	"Core"	Main "core" peptide
Lys	3.0	0
His	2.8	0
Arg	1.9	0
Asp	6.7	5.0
Thr	5.1	4.2
Ser	2.2	3.2
Glu	19.9	31.8
Pro	3.0	7.4
Gly	5.8	2.7
Ala	7.9	9.3
Val	6.4	0
Met (sulphone)[b]	4.5	3.4
Ile	8.3	11.5
Leu	8.0	11.4
Tyr	2.7	2.8
Phe	7.8	7.3
Cys[c]	1.7	0

[a] Expressed as moles %, as the molecular weight of the material used is not known.

[b,c] The material was performic acid oxidized before digestion by trypsin.

less than half the number expected has been found, again suggesting that phosphonatase is composed of two identical subunits.

Some Properties of Phosphonatase

Phosphonatase showed optimal activity between pH 8 and 9 (Fig. V.10). The rate of release of Pi from 2-phosphonoacetaldehyde was directly proportional to the concentration of enzyme over the range tested (Fig. V.11). The apparent Michaelis constant (K_m) for phosphonatase, estimated by measuring the release of Pi and of acetaldehyde from 2-phosphonoacetaldehyde, was about 4×10^{-5} M (Fig. V.12), which is comparable to that of alkaline phosphatase at dilute substrate concentrations (Garen and Levinthal, 1960; Heppel, Harkness and Hilmoe, 1962; see also Discussion). The activity of phosphonatase is inhibited by increasing concentrations of NaCl (Table V.F), whereas that of alkaline phosphatase is stimulated by increasing ionic strengths (Plocke and Vallee, 1962).

Phosphonatase was unable to cleave AEP, aminomethylphosphonate, α-, β-glycerophosphate and ethanolaminephosphate, although it did cleave p-nitrophenylphosphate slowly (Table V.G). The shock-fluid from a strain of E. coli (Chou and Neu, 1967), containing

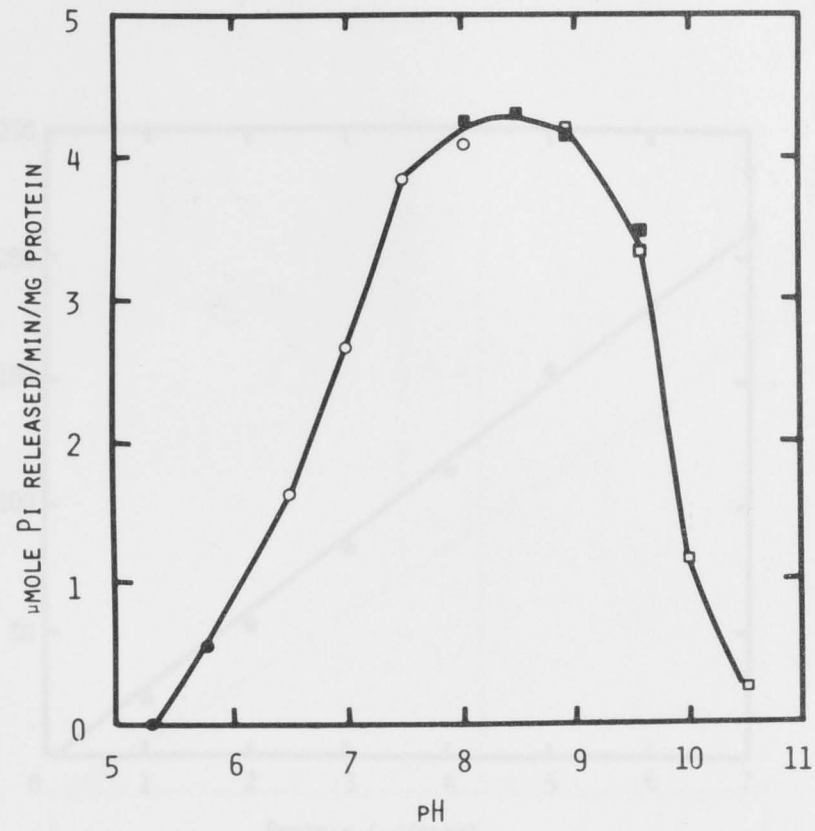


Fig. V.10. The effect of pH on the activity of phosphonatase. The assay conditions are the same as those described in Materials and Methods, except that the pH of the reaction mixture was varied by using a series of different buffers, all at 50 mM : sodium acetate, ●——● ; TES-HCl, ○——○ ; Ammediol-HCl, ■——■ ; glycine-KOH, □——□ . The enzyme reaction was followed by measuring the release of Pi from 2-phosphonoacetaldehyde which is also described in Materials and Methods.

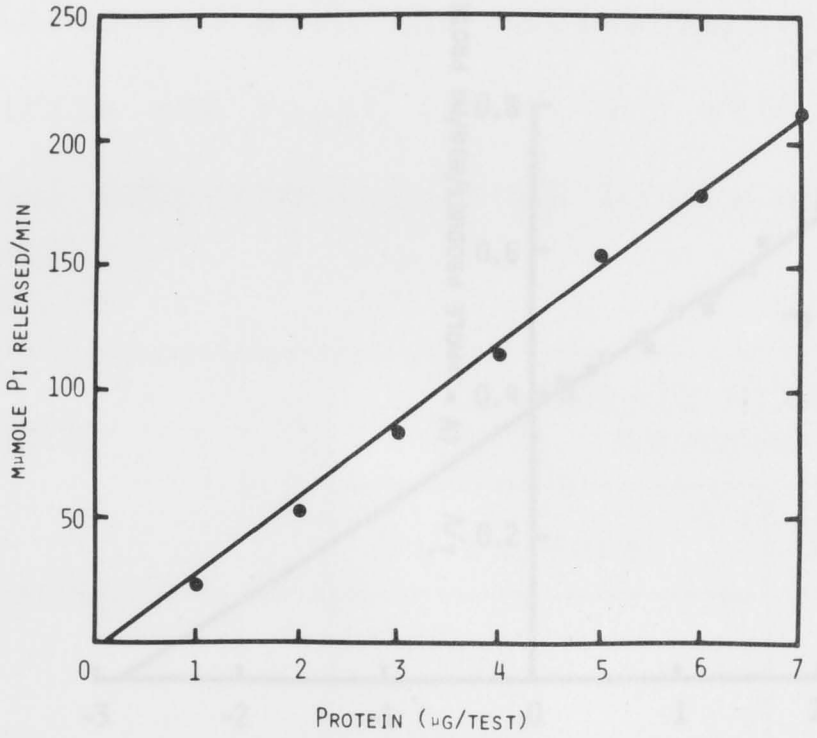


Fig. V.11. The effect of enzyme concentration on the activity of phosphonatase. The assay conditions and the method for estimating the amount of Pi released from 2-phosphonoacetaldehyde are described in Materials and Methods.

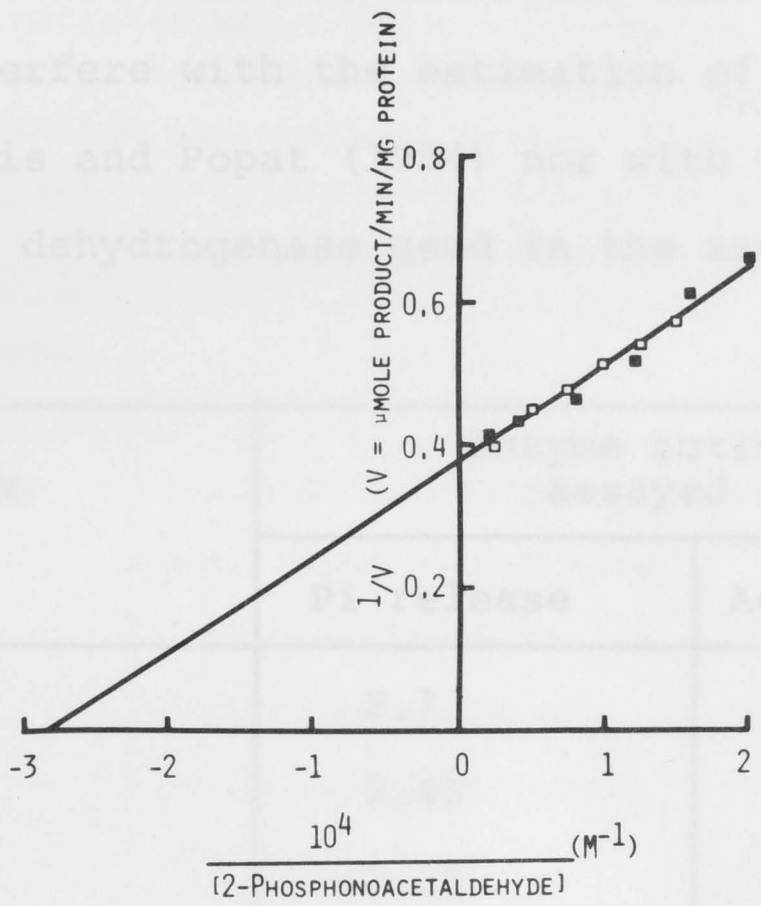


Fig. V.12. Determination of the apparent Michaelis constant (K_m) of phosphonatase for 2-phosphonoacetaldehyde. The conditions of assay, and the methods used for determining the amount of Pi (□——□) or acetaldehyde (■——■) released from 2-phosphonoacetaldehyde, are described in Materials and Methods. The K_m was estimated to be about 4×10^{-5} M.

Table V.F. The effect of alterations in the ionic environment on the activity of phosphonatase

The conditions of assay are as described in Materials and Methods, except that varying concentrations of NaCl were added as indicated below. NaCl (1 M) did not interfere with the estimation of Pi by the method of Harris and Popat (1954) nor with the activity of the alcohol dehydrogenase used in the assay for acetaldehyde.

[NaCl]M	Enzyme activity (units/mg) assayed as :	
	Pi release	Acetaldehyde release
0	2.7	2.29
0.05	2.45	1.56
0.125	2.18	1.29
0.25	1.65	1.29
0.50	0.90	0.83
1.00	0.40	0.66

Table V.G. Substrate-specificity of phosphonatase

Phosphonatase (4 μg/ml) was incubated at 25° in 50 mM Ammediol-HCl buffer (pH 8.5) containing 5 mM $MgCl_2$ and various potential substrates shown below. The amount of Pi released was determined as described in Materials and Methods. The action of alkaline phosphatase, present in the shock-fluid[a,b] from E. coli (33 μg/ml), is shown for comparison.

Enzyme tested	Compound	Activity (units/mg)
Phosphonatase	2-phosphonoacetaldehyde	4.64
	p-nitrophenylphosphate	0.15[c]
	α-glycerophosphate	0[d]
	β-glycerophosphate	0[d]
	ethanolamine phosphate	0[d]
	AEP	0[d]
	aminomethylphosphonate	0[d]
E. coli shock-fluid (containing alkaline phosphatase)	β-glycerophosphate	0.47
	2-phosphonoacetaldehyde	0[d]

[a] Chou and Neu (1967).

[b] Kindly supplied by Mr. N. Medveczky.

[c] This reaction was not inhibited by 2.5 mM orthophosphite.

[d] Incubated for 1 h.

alkaline phosphatase activity, did not degrade 2-phosphonoacetaldehyde, even after incubation for 1 h with the compound.

Phosphonatase is heat-labile as, even in the presence of 5 mM Mg^{++}, its optimal activity is at 45° (Fig. V.13). The activation energy, calculated from an Arrhenius plot of the data for Fig. V.13 is 9.5 Kcal/mole (Fig. V.14). The turnover number for freshly purified enzyme is about 660 molecules of 2-phosphonoacetaldehyde cleaved/enzyme molecule/min at 25°, as estimated for a molecular weight of 75,000 daltons. This is approximately one-quarter the turnover number of alkaline phosphatase (Garen and Levinthal, 1960).

The isoelectric point was estimated roughly by adding a small sample of the purified enzyme to a series of buffers set 0.5 pH units apart, ranging from pH 3.5 to pH 7.5. A precipitate formed within 10 min in the buffer at pH 4.5, and a slight precipitate formed overnight at pH 5.0, indicating that the isoelectric point is in the region of pH 4.6.

Preliminary experiments indicate that metals other than Mg^{++} may be necessary for activity, since NaCN (10^{-2} M) totally inhibited the release of Pi

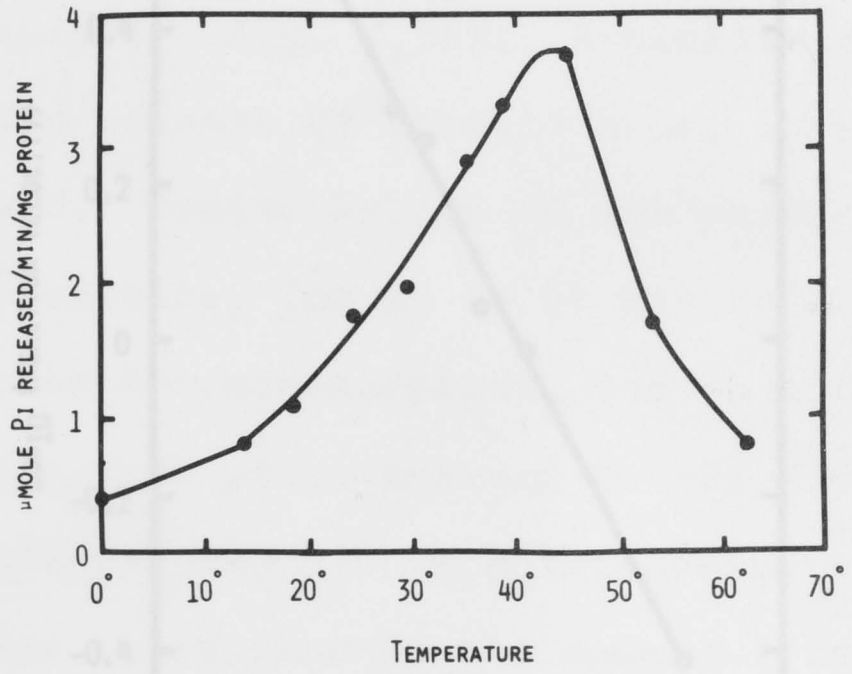


Fig. V.13. The effect of temperature on the activity of phosphonatase. The assay conditions and the method for estimating the amount of Pi released from 2-phosphonoacetaldehyde are described in Materials and Methods. The enzyme concentration was 5 μg/ml.

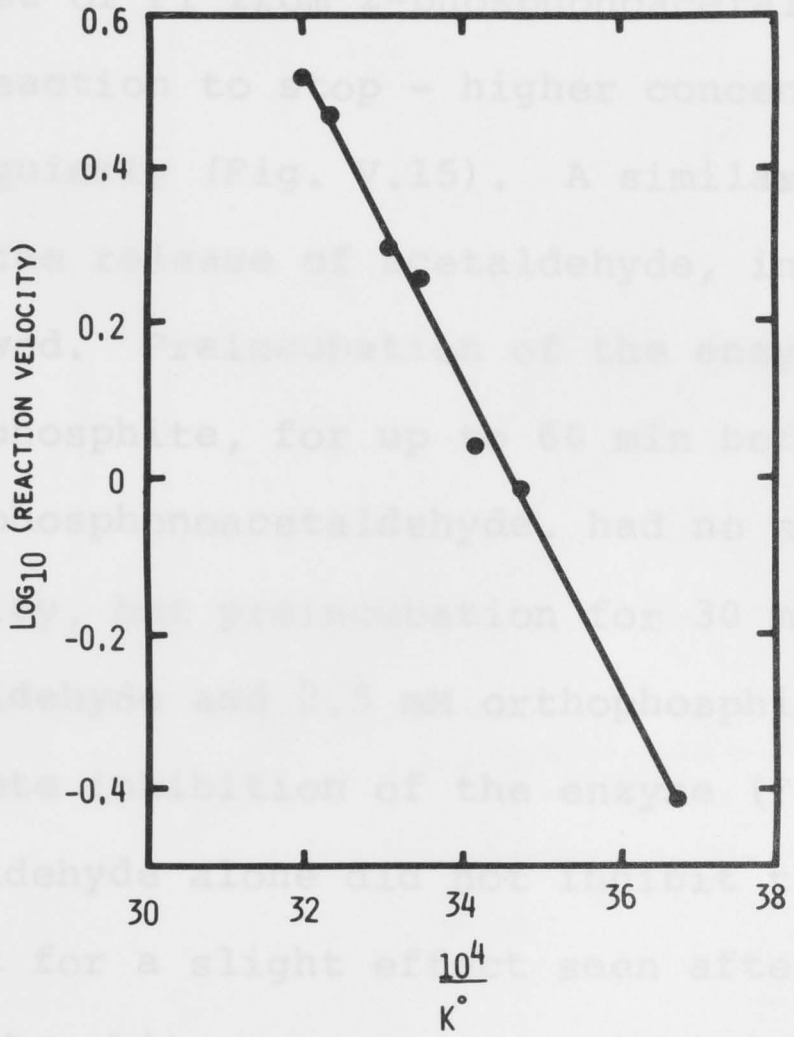


Fig. V.14. The effect of temperature on the activity of phosphonatase - Arrhenius plot. The data, used in Fig. V.13, were plotted as $\log_{10}$ (reaction velocity) v. the reciprocal of the absolute temperature (°K). The apparent activation energy was estimated to be about 9.5 kcal/mole of 2-phosphonoacetaldehyde.

from 2-phosphonoacetaldehyde, although NaF (1 mM, without Mg^{++} added to the assay system) had no effect.

The Effect of Orthophosphite on the Activity of Phosphonatase

Orthophosphite had a marked effect on the rate of release of Pi from 2-phosphonoacetaldehyde, as it caused the reaction to stop - higher concentrations doing so more quickly (Fig. V.15). A similar result was obtained when the release of acetaldehyde, instead of Pi, was followed. Preincubation of the enzyme with 2.5 mM orthophosphite, for up to 60 min before the addition of 2-phosphonoacetaldehyde, had no effect on subsequent activity, but preincubation for 30 min with 25 mM acetaldehyde and 2.5 mM orthophosphite resulted in complete inhibition of the enzyme (Fig. V.16); acetaldehyde alone did not inhibit the reaction, except for a slight effect seen after 20 min. Thus, orthophosphite can act as an inhibitor only in the presence of 2-phosphonoacetaldehyde or acetaldehyde. Prolonged dialysis of orthophosphite-inactivated enzyme resulted in recovery of only 30-40% of the activity (Table V.H).

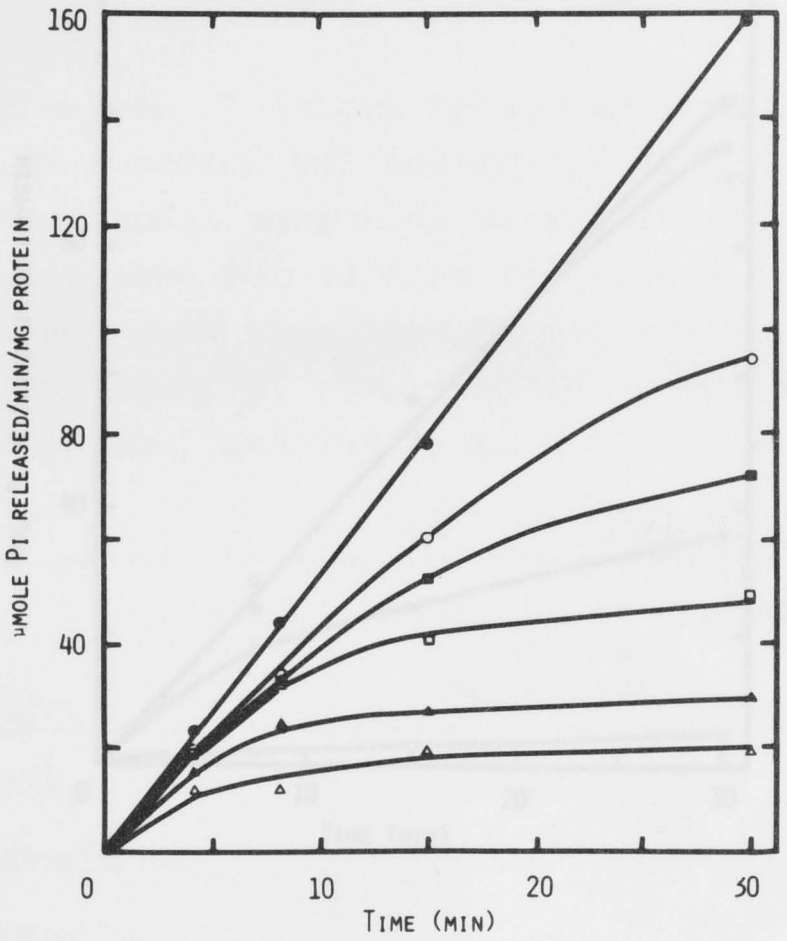


Fig. V.15. The effect of varying concentrations of orthophosphite on the activity of phosphonatase. Varying concentrations of orthophosphite were added to the reaction mixture, and the release of Pi estimated as described in Materials and Methods. The concentrations of orthophosphite used were : 0, ●—● ; 0.5 mM, ○—○ ; 1 mM, ■—■ ; 2 mM, □—□ ; 4 mM, ▲—▲ ; 8 mM, △—△ .

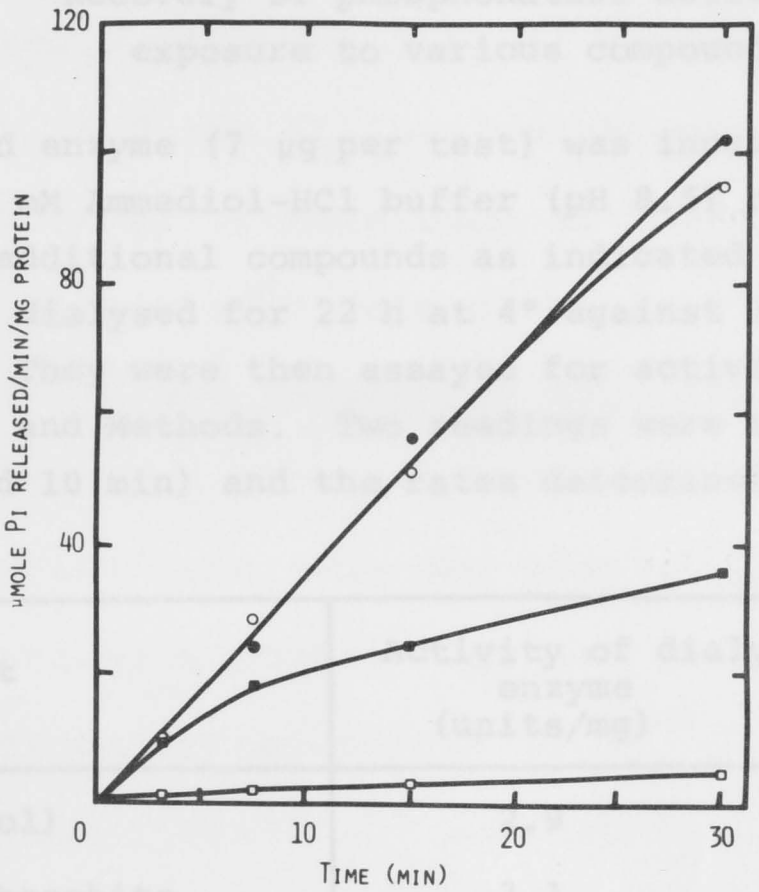


Fig. V.16. The effect on the activity of phosphonatase of pre-incubation with various compounds. The assay conditions described in Materials and Methods were used. Phosphonatase was incubated for 30 min in the absence of 2-phosphonoacetaldehyde with no additions (●—●), 25 mM acetaldehyde (○—○), 2.5 mM orthophosphite (■—■), and 25 mM acetaldehyde plus 2.5 mM orthophosphite (□—□). At zero time, 2 mM 2-phosphonoacetaldehyde was added and the release of Pi followed.

Table V.H. Recovery of phosphonatase activity following exposure to various compounds

Purified enzyme (7 µg per test) was incubated for 30 min at 25° in 50 mM Ammediol-HCl buffer (pH 8.5) containing 5 mM $MgCl_2$, with additional compounds as indicated below. The samples were dialysed for 22 h at 4° against 3 x 200 ml of TMD buffer. They were then assayed for activity as described in Materials and Methods. Two readings were taken for each sample (5 and 10 min) and the rates determined.

Treatment	Activity of dialysed enzyme (units/mg)	% Control
None (control)	2.9	100
5 mM orthophosphite	3.1	107
25 mM acetaldehyde	3.2	110
5 mM orthophosphite + 2 mM 2-phosphonoacetaldehyde	0.9	31
5 mM orthophosphite + 25 mM acetaldehyde	1.2	42

DISCUSSION

An enzyme able to cleave the carbon-phosphorus bond has not been reported before. The enzyme described here has been called "phosphonatase" and resembles the alkaline phosphatase of E. coli in a number of its properties. It shows optimal activity over a similar pH range, and its K_m for 2-phosphonoacetaldehyde is of the same order of magnitude as that of alkaline phosphatase at low substrate concentrations (Garen and Levinthal, 1960; Heppel et al., 1962)[a]. Preliminary experiments indicate that its molecular weight is approximately the same and that it also is composed of two identical subunits (Rothman and Byrne, 1963). Both enzymes are protected by Mg^{++}, and inactivated by EDTA, CN^- (Garen and Levinthal, 1960; Plocke, Levinthal and Vallee, 1962), but not by F^- (Heppel et al., 1962; Rosenkranz, Bendich and Beiser, 1963).

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[a]Heppel et al. (1962) found that the Lineweaver-Burk plot for alkaline phosphatase gave two intersecting lines. Measurements at dilute substrate concentrations gave a K_m of the order of 10^{-5} M (1.4×10^{-5} M for p-nitrophenylphosphate and 3.3×10^{-5} M for AMP). However, when the substrate was present at relatively high concentrations, the K_m values obtained were of the order of 10^{-3} M.

The effect of these metal complexing agents suggests that phosphonatase may require certain metals (perhaps Zn^{++} or Co^{++}) to maintain its tertiary structure (cf. Simpson, Vallee and Tait, 1968), but there is not yet enough data to suggest which ones these might be. Studies with the wild strain of B. cereus (W) (see Chapter II), indicate that it is metabolically costly for the cell to use AEP as a sole source of phosphorus, since its entry is strictly controlled. The formation of the transport system for AEP is severely repressed by Pi. The formation and activity of alkaline phosphatase by E. coli is also repressed by Pi (Torriani, 1960), although the mechanism of inhibition is probably different, as alkaline phosphatase lies outside the plasma membrane (Done, Shorey, Loke and Pollak, 1965).

Phosphonatase, however, is not an alkaline phosphatase. It is unable to degrade several phosphate-esters which are readily degraded by alkaline phosphatase, with one exception - p-nitrophenylphosphate - and this is at one-thirtieth the rate at which 2-phosphonoacetaldehyde is catabolised. Conversely, a crude preparation of alkaline phosphatase from E. coli was unable to degrade 2-phosphonoacetaldehyde, even after prolonged exposure to the compound. Preliminary

experiments indicate that phosphonatase is not markedly inhibited by concentrations of Pi up to 0.1 M. Pi, on the other hand, is an effective competitive inhibitor of alkaline phosphatase activity against p-nitrophenyl-phosphate (K_i = 0.38 x 10^{-5} M; Garen and Levinthal, 1960).

Alkaline phosphatase attacks a wide range of phosphate-esters, but surprisingly, it hydrolyses these all at about the same rate (Garen and Levinthal, 1960). Wilson, Dyan and Cyr (1964) found that Tris could be extensively phosphorylated by alkaline phosphatase if the concentrations were high (about 1 M). To explain these observations, they suggested that hydrolysis proceeds through a common phosphorylated-enzyme complex which can donate Pi either to water or to Tris, and that the dephosphorylation of the complex is the rate-controlling step in the process. Recent kinetic studies of Barrett, Butler and Wilson (1969) have confirmed that a phosphorylated-enzyme complex is a true catalytic intermediate in this reaction, although other workers have shown that above pH 8, it is not the dephosphorylation of the complex that is rate-limiting, but some other process concerned with its formation, such as rearrangement of the enzyme of the

enzyme-substrate complex (Aldridge, Barman and Gutfreund, 1964; Fernley and Walker, 1966). Thus, the sequence of reactions catalysed by alkaline phosphatase may be represented as follows :

$$ROP + E \rightleftharpoons ROP — E \overset{H_2O}{\rightleftharpoons} P — E^* \rightleftharpoons P — E \rightleftharpoons Pi + E + ROH$$

where E = alkaline phosphatase

E* = altered form of this enzyme

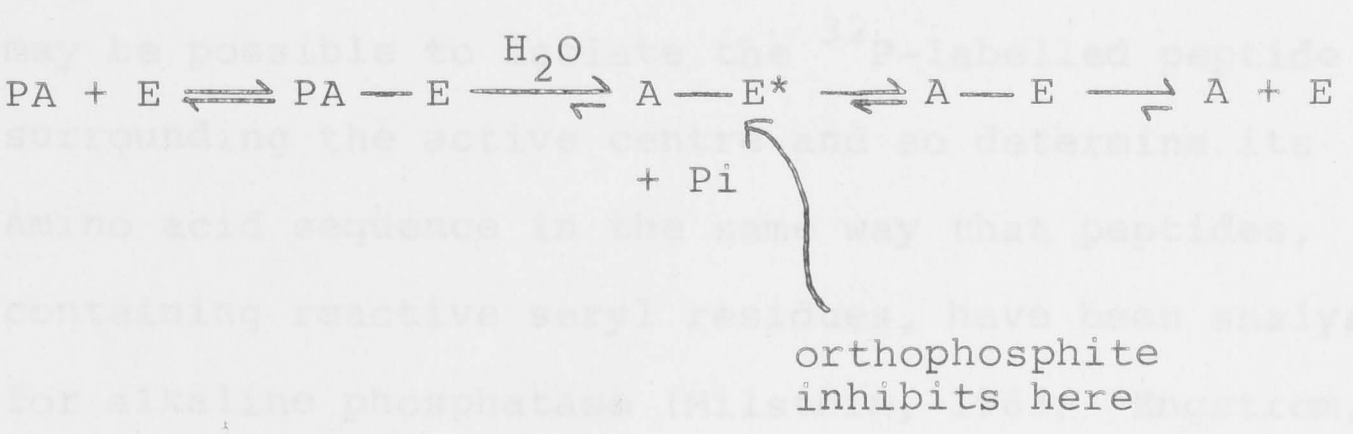
ROP = phosphate-ester

In contrast, the range of substrates degraded by phosphonatase seems to be very limited. The cleavage of the carbon-phosphorus bond in alkaline solutions requires that the electrons of the bond be accommodated by a neighbouring, electron-withdrawing group (see Clark et al., 1964, in the Discussion to Chapter IV). However, 2-phosphonoacetaldehyde is stable in aqueous solutions at 25°, both at high and low pH values (Isbell, Englert and Rosenberg, 1969), although at higher temperatures it is hydrolysed much more rapidly than AEP. The work with the inhibitor orthophosphite (discussed below) suggests that both the phosphonate and the acetaldehyde moieties of 2-phosphonoacetaldehyde

may combine with the enzyme (perhaps the former through a seryl residue and the latter through a histidyl or lysyl residue), so enabling the necessary electron shifts to take place far more easily. The reason why p-nitrophenylphosphate, but not other phosphate-esters, is cleaved by phosphonatase may reside in the strong electron-withdrawing group (the nitrophenyl group) which may substitute for that part of the enzyme which normally is attached to the acetaldehyde moiety. In contrast to the situation when 2-phosphonoacetaldehyde is the substrate, orthophosphite does not inhibit the release of Pi from p-nitrophenylphosphate, which suggests that a different reaction mechanism is operating. Trentham (1969) has found that 2-hydroxy-5-nitrobenzylphosphonate is an effective competitive inhibitor for alkaline phosphatase, but that it is not cleaved by the enzyme. I have not yet been able to test whether phosphonatase can degrade this compound.

The inhibition of phosphonatase by orthophosphite is effectively irreversible. The structural similarity of orthophosphite to the phosphonate moiety of 2-phosphonoacetaldehyde suggests that it acts at the active site, particularly as it acts as an inhibitor only in the presence of the substrate (2-phosphono-

acetaldehyde) or one of the products (acetaldehyde). One explanation of this is that the release of products is ordered : only 2-phosphonoacetaldehyde or acetaldehyde can react with the free enzyme. When 2-phosphonoacetaldehyde is the substrate, Pi is the first product to be released, leaving acetaldehyde attached to the enzyme surface. The conformational state of the enzyme-acetaldehyde complex is different from that of the free enzyme, and orthophosphite, by its structural analogy to Pi, combines with this form of the enzyme to produce an irreversible complex :



E = phosphonatase

E* = altered form of this enzyme

PA = 2-phosphonoacetaldehyde

As yet, I have been unable to test by kinetic means whether or not the release of products is ordered. This reaction is not readily reversible and I have found that concentrations up to 0.1 M of acetaldehyde

and Pi have to be used for any product inhibition to be observed. It is difficult to assess the side effects that such concentrations could be having on the activity of the enzyme, especially since phosphonatase, in contrast to alkaline phosphatase (Plocke and Vallee, 1962), is adversely affected by increasing ionic strengths. I have no evidence yet that orthophosphite is bound to phosphonatase, and if so, whether it is bound in a Michaelis-type complex or covalently to the enzyme. If the latter is the case, then, by using [^{32}P]orthophosphite, it may be possible to isolate the ^{32}P-labelled peptide surrounding the active centre and so determine its amino acid sequence in the same way that peptides, containing reactive seryl residues, have been analysed for alkaline phosphatase (Milstein, 1963; Engstrom, 1964; Schwartz, Crestfield and Lipmann, 1963) and the diisopropylphosphorofluoridate-sensitive enzymes (see Dixon and Webb (1964) p.474, for a summary). Trentham (personal communication) has suggested an alternative mechanism to explain the action of orthophosphite. He suggested that orthophosphite might be acting non-specifically by reducing a Schiff's base form of the acetaldehyde-enzyme complex in a similar

manner to borohydride. In this case, the inactive enzyme would not contain any radioactivity from [^{32}P]orthophosphite, but would incorporate label from [^{14}C]acetaldehyde.

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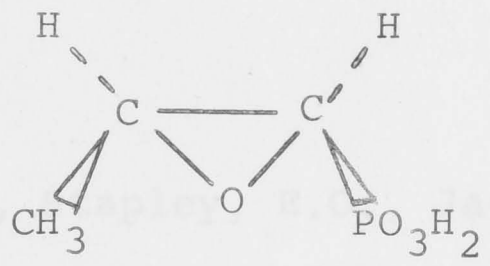
ADDENDUM

After this thesis had been completed, a group of workers from Merck Sharp and Dohme (for references, see below) reported the isolation and identification of a new, naturally-occurring phosphonate. The compound, named "phosphonomycin", is an antibiotic produced by several species of streptomycetes. It has been identified as 1,2-epoxypropylphosphonic acid :



Phosphonomycin

Its structure has been confirmed by comparison with synthetic material.

Phosphonomycin is effective orally in protecting mice against a number of infections caused by Gram-positive and Gram-negative organisms, and it appears to be non-toxic to both mice and man. It enters the bacteria via the transport system for α-glycerophosphate. Once inside the cells, it combines covalently and irreversibly with pyruvate-uridine diphospho-N-acetylglucosamine transferase, an enzyme which catalyses the first step in the biosynthesis of nucleotide muramyl peptides, which are cell-wall precursors in many bacteria. Thus, its overall effect

on the bacteria is similar to that of penicillin.

If the use of phosphonomycin becomes widespread, it will be interesting to see whether resistant bacteria appear in the community. One obvious means for the bacteria to become resistant is for them to fail to transport the antibiotic. Another is for them to produce an enzyme (similar to penicillinase) which cleaves phosphonomycin; perhaps phosphonatase may be able to catalyse this reaction.

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